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Mass Spectrometry Library of NPS: Isolation and Characterisation of Designer Drugs from Herbal Incenses and Plant Feeders

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Dissertação

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Abstract

For the past ten years, a growing spread of new psychoactive substances (NPS) has been witnessed worldwide. In order to circumvent existing narcotics and psychotropic substances legislations, these compounds have been traditionally sold as innocuous products, advertised as being herbal incenses, plant feeders or bath salts, in smartshops or over the Internet.

The rapidly growing problem of NPS makes international control a real challenge, with the traditional detection methods becoming increasingly inadequate. In Portugal, the identification of these compounds became more important with the implementation of a new decree in 2013 (*Dec-Lei 54/2013*, of 17th of April), which forbids the production and commercialisation of about 159 NPS, being liable to fast updates. In order to create effective analytical databases to facilitate the identification of these drugs in Portugal, a collaboration was established between *Faculdade de Ciências da Universidade de Lisboa* (FCUL) and the *Laboratório de Polícia Científica da Polícia Judiciária* (LPC/PJ). With the decree, more than 34000 products that were sold in 8 Portuguese smartshops were delivered to LPC/PJ. From those, two types of products, plant feeders and herbal incenses, known to contain two of the most reported categories of NPS, synthetic cathinones and synthetic cannabinoids, were selected for analyses. The plant feeders and herbal incenses were analysed in order to create an in-house Gas Chromatography-Electron Impact-Mass Spectrometry (GC-EI-MS) library of NPS, as their characterisation and structural elucidation by Nuclear Magnetic Resonance Spectroscopy (NMR) will allow their use as secondary standards in routine GC-MS analysis at LPC/PJ.

In this project, the methanolic extracts of 169 samples (111 plant feeders, 56 herbal incenses and 5 seized products) were studied. The identification of 26 NPS (12 synthetic cathinones, 12 synthetic cannabinoids, 5-MeO-DALT and ethylphenidate) was achieved by EI-MS fragmentation patterns of all the compounds and NMR characterisation of mephedrone, ethylphenidate, JWH-018, JWH-122, JWH-210 and SGT-25. EI-MS was based on the characteristic fragments of each compound and NMR was based on one and two-dimensional experiments. This study led to the unequivocal identification of 21 secondary standards that allowed the creation of an in-house library of NPS.

The first batch of secondary standards analysed by GC-EI-MS in LPC/PJ were 8 plant feeders and two synthetic compounds that contained 10 cathinones (3,4-DMMC, 4-MEC, buphedrone, flephedrone, MDBPB, MDPV, methedrone, methylone, *N*-ethylcathinone and pentedrone) explicitly identified in FCUL by NMR and GC-EI-MS previously in this project. This EI-MS library was used in the study of 103 plant feeders with 15 different brand names and 16 different lot numbers, as to assess their qualitative and quantitative variability within lots and brand names. Buphedrone and MDPBP were not detected in these samples, but a compound not yet present in the library was detected, ethylphenidate. In some of those plant feeders, caffeine was also detected. Analysis of previous results from seized plant feeders analysed at LPC/PJ in 2011 permitted the inclusion of mephedrone in the database, after confirmational analysis by NMR.

In the GC-EI-MS analyses of 53 herbal incenses, 11 synthetic cannabinoids, one cathinone (MDPV), Vitamin E, caffeine and a tryptamine derivative, 5-Meo-DALT, were identified. Seven out of the eleven detected cannabinoids were isolated from herbal incenses (JWH-018, JW-122 and JWH-210 in this project). All the 7 purified cannabinoids (JWH-018, JWH-122, JWH-210, AKB48, UR-144, XLR-11 and MAM2201) were clearly identified by NMR permitting their use as secondary standards.

The developed methodology was applied in 4 seized samples, allowing the identification of two NPS not included in the library: SGT-25, a synthetic cannabinoid recently reported in the European Union (EU); and 4F-PBP, a novel synthetic cathinone in the EU reported by us during this project.

This project allowed the construction of an in-house GC-EI-MS library of synthetic NPS, comprising 21 compounds in total: 12 cathinones (3,4-DMMC, 4F-PBP, 4-MEC, buphedrone, flephedrone, MDBPB, MDPV, mephedrone, methedrone, methylone, *N*-ethylcathinone and pentedrone), 8 cannabinoids (AKB48, JWH-018, JWH-122, JWH-210, MAM2201, UR-144, SGT-25 and XLR-11) and ethylphenidate.

Keywords: GC-EI-MS library; NMR; Synthetic Cathinones; Synthetic Cannabinoids; NPS

Resumo

Nos últimos anos tem-se verificado uma crescente propagação de novas substâncias psicoativas (NSP) em todo o mundo. Só na Europa, em 2014, mais de 100 novas NSP foram reportadas. NSP são definidas pelo Conselho Europeu como "*substâncias em estado puro ou numa preparação que podem constituir uma ameaça à saúde pública comparável às substâncias listadas nas convenções das Nações Unidas"*. Estes compostos têm sido tradicionalmente vendidos em *smartshops* ou na Internet como produtos inócuos, anunciados como sendo incensos herbais, fertilizantes para plantas ou sais de banho, sempre com a informação de que não se destinam ao consumo humano.

O problema do rápido desenvolvimento destas substâncias cria grandes desafios ao nível do controlo internacional, em que as metodologias tradicionais de análise mostram-se, por vezes, insuficientes face a estes novos compostos. Em Portugal, a identificação de NSP ganhou especial relevo com a entrada em vigor do Decreto-Lei 54/2013, de 17 de Abril, que proíbe, entre outros, a produção e comercialização de cerca de 159 NSP, sendo passível de rápidas atualizações, de modo a manter-se a par com o diário aparecimento de novas substâncias. Em Junho de 2014, um protocolo foi assinado entre a Faculdade de Ciências da Universidade de Lisboa (FCUL), o Laboratório de Polícia Científica da Polícia Judiciária (LPC/PJ) e a Faculdade de Farmácia da Universidade do Porto (FFUP), com o objetivo de criar bases de dados eficazes de modo a facilitar a identificação deste tipo de compostos em Portugal e avaliar a sua toxicidade.

Este trabalho consistiu na análise de incensos herbais e fertilizantes para plantas de 8 *smartshops* da Área Metropolitana de Lisboa, entregues voluntariamente aquando da entrada em vigor do Decreto-Lei 54/2013 ou de produtos apreendidos. A metodologia desenvolvida neste trabalho consiste na análise destes produtos por Cromatografia Gasosa acoplada à Espectrometria de Massa (GC-MS) e Espetroscopia de Ressonância Magnética Nuclear (RMN), com o objetivo de caracterizar os compostos presentes e permitir o seu uso enquanto padrões qualitativos secundários em análises de rotina por GC-MS no LPC/PJ.

Uma compilação dos produtos de acordo com a *smartshop* de origem, o nome do produto e o lote permitiu aferir que no total, 34649 produtos foram entregues voluntariamente. Destes, 6374 eram fertilizantes para plantas em pó e 15177 eram incensos herbais.

Fertilizantes para plantas e incensos herbais têm, geralmente, na sua constituição, catinonas sintéticas e canabinóides sintéticos, duas das categorias de compostos mais reportadas no espaço europeu.

Inicialmente, 8 fertilizantes para plantas e 2 produtos de síntese previamente analisados por GC-MS e RMN na FCUL, foram analisados por GC-EI-MS no LPC/PJ, permitindo a construção de uma primeira biblioteca de espectros de massa com 10 catinonas sintéticas: 3,4-DMMC, 4-MEC, bufedrona, flefedrona, MDBPB, MDPV, metedrona, metilona, *N*-etilcatinona e pentedrona. Esta análise permitiu a caracterização de fragmentos mássicos característicos para cada composto e para substituições características e passíveis de ocorrerem neste tipo de compostos. De modo a caracterizar qualitativa e quantitativamente fertilizantes para plantas vendidos em Portugal com o mesmo nome e o mesmo número de lote, 103 fertilizantes para plantas com 15 nomes diferentes (Blast, Bliss, Bloom, Blow, Charlie, Crabby, Cyclop, Darko, Demon, E.T., Kick, Mush, Rush, The Cannon, Vamp) e 16 números de lote distintos, foram analisados, sendo a sua identificação baseada na comparação com a base de dados criada. Verificou-se uma uniformidade qualitativa dos produtos, no que ao número de lote diz respeito. Das 10 catinonas presentes na biblioteca, não foram detetadas a bufedrona e a MDPBP nestes lotes de amostras. Desta análise, detetou-se um composto que não constava da base de dados *in-house*, cuja análise por GC-MS sugeriu tratar-se do etilfenidato. A sua análise por RMN permitiu a caracterização estrutural do composto, confirmando a identificação efectuada por espectrometria de massa. Em alguns dos fertilizantes, foi também detetada a cafeína, um adulterante comum neste tipo de produtos.

Com o intuito de obter mais padrões secundários de NSP, fez-se um levantamento de resultados de análises a fertilizantes para plantas realizados no LPC em 2011. Uma amostra do fertilizante para plantas “Blow”, cuja análise por GC-MS revelou a presença de mefedrona, foi analisada por RMN, que

confirmou a identificação realizada e permitiu a inclusão deste composto na base de dados.

Uma análise preliminar por GC-MS de 33 incensos herbais (2012, Algerian Blend, Apple, Atomic Bomb, Blow, Bombastic Kaboom Spliff Atomic Bomb, B.R.O.S., Buddah, Butterfly, Caramba, Cheese, Esfinge, Freemind, Future, Home Run, Kaboom, Magic, Mandala, MÁUI, Maya2012, Planet H, PUM!, Radioactive, Rainbow, Red Sunshine, Royal Mix, Smoke, So High, Spike99, Spliff, T-Rex, The Unicorn, Tornado, Whacked), permitiu a identificação, por comparação com a biblioteca de espectros SWGDRUG, de 10 canabinóides sintéticos (JWH-018, JWH-122, JWH-210, JWH-250, MAM2201, AKB48, UR-144, AM694, AM684-derivado cloro e AM1248). Um estudo das fragmentações de massa de cada composto possibilitou aferir fragmentações características para diferentes grupos estruturais, permitindo uma futura análise mais simplificada destes compostos. Uma segunda análise por GC-MS foi realizada em incensos herbais, de modo a seleccionar produtos para isolamento dos compostos da matriz herbal por cromatografia líquida e sua caracterização por RMN, de modo a obter o maior número de padrões secundários possível. Desta análise identifcou-se um outro composto, XLR-11 (derivado fluorado do UR-144). Neste âmbito, foram isolados da matriz por cromatografia líquida, e analisados por RMN, 7 canabinóides sintéticos JWH-018, JWH-122, JWH-210, AKB48, UR-144, XLR-11 e MAM2201, que foram usados para a construção de uma base de dados *in-house* para canabinóides sintéticos.

A relevância da base de dados criada foi comprovada com a identificação de uma nova NPS nunca antes reportada no espaço europeu, 4F-PBP. A presença de fragmentações de massa características permitiu a sugestão de uma estrutura para o composto, que foi elucidada por RMN, permitindo a inequívoca identificação desta nova catinona sintética. A análise por RMN permitiu ainda a deteção de um outro composto, mio-inositol, não detetado por GC-MS. A construção desta base de dados permitiu a identificação de um novo composto, que, aquando da altura da análise, ainda não se encontrava presente nas bibliotecas de espectros de massa existentes no LPC/PJ. O composto foi analisado por GC-MS e RMN, tendo sido

identificado como tratando-se do SGT-25, um canabinóide sintético recentemente reportado na Europa. A escassez de dados analíticos deste composto levou ao seu estudo de fragmentações mássicas e análise por NMR em diferentes solventes, $CDCl_3$, MeOD, DMSO e benzeno (d_6).

Este trabalho permitiu a construção de uma biblioteca de espectros de massa e tempos de retenção *in-house* composta por 21 compostos sintéticos: 12 catinonas sintéticas (3,4-DMMC, 4F-PBP, 4-MEC, bufedrona, flefedrona, MDPBP, MDPV, mefedrona, metedrona, metilona, *N*-etilcatinona e pentedrona), 8 canabinóides sintéticos (JWH-018, JWH-122, JWH-210, AKB48, UR-144, MAM2201, XLR-11 e SGT-25) e o etilfenidato (Figura 1). Estes resultados comprovam a aplicabilidade da metodologia proposta neste trabalho na análise de NPS, permitindo uma mais fácil e mais eficaz análise de rotina por parte de um laboratório forense, cuja resposta quer-se rápida e adequada ao fim a que se destina.

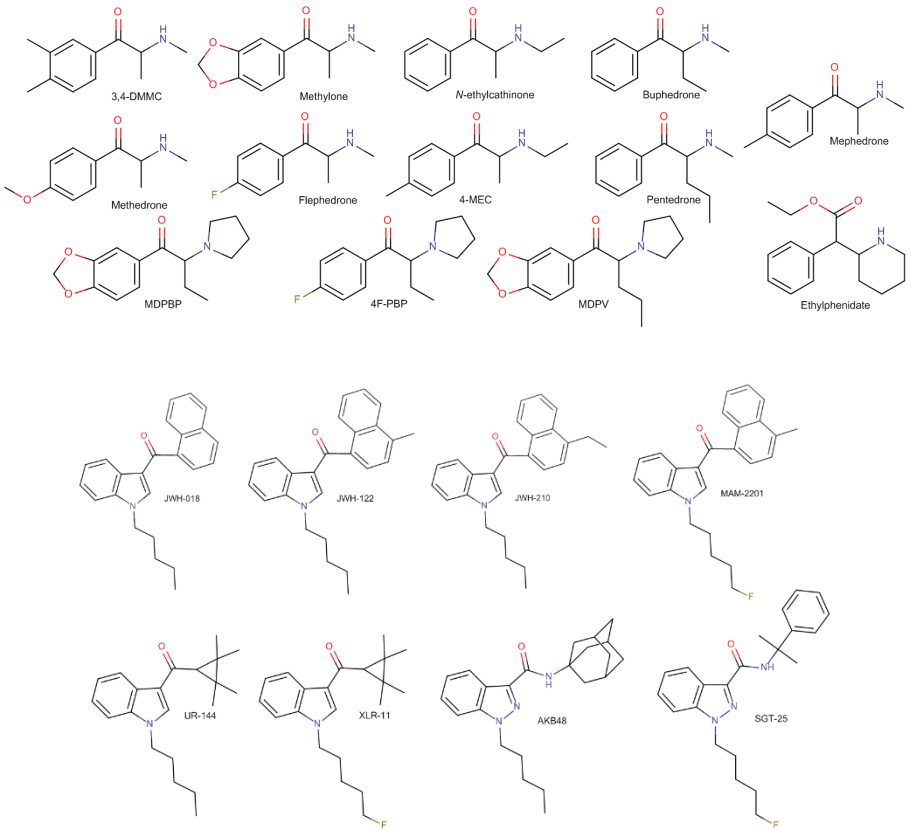

Figura 1 - Biblioteca de EI-MS de Catinonas Sintéticas e Canabinóides Sintéticos

Palavras-chave: Biblioteca GC-EI-MS; NMR; Catinonas Sintéticas; Canabinóides Sintéticos

Publication of Results

Part of the original results obtained within the scope of this project have been published in the scientific community or in international conferences. Press release from FCUL led to the appearance on several national newspapers.

Scientific Journal

Gaspar, H., Bronze, S, Ciríaco, S., **Queirós, C.R**., Matias, A., Rodrigues J., Oliveira, C., Cordeiro, C. and Santos, S., "*4F-PBP: 4F-PBP (4'-fluoro-α-pyrrolidinobutyrophenone), a new substance of abuse: structural characterization and purity NMR profiling"*, Forensic Science International, 252, pp. 168-176, 2015 - Appendix I

Communications

Leal, C., Lopes, R., Matias, A., Rodrigues, J. and Gaspar, H., "Identification of Synthetic Cathinones in Plant Feeders", *II Jornadas Ibéricas de Toxicologia, 13-15 November 2014, Faculdade de Ciências da Saúde, Universidade da Beira Interior*, Covilhã, Portugal (Poster) - Appendix II

Leal, C., Ciríaco, S., Matias, A. and Gaspar, H. "Characterisation of Plant Feeders", *3rd International Meeting on Forensic Science and Criminal Behaviour - Globalization of Crime*, *8-9 May 2015, Instituto Superior de Ciências da Saúde Egas Moniz,* Almada, Portugal (Oral) - Appendix III

Queirós, C., Gonçalves, C., Ciríaco, S., Matias, A., Rodrigues, J. and Gaspar, H., "NMR Characterisation of SGT-25: A New Psychoactive Substance", *19th European Symposium on Organic Chemistry, Faculdade de Ciências da Universidade de Lisboa,* 12-16 July 2015, Lisboa, Portugal (Poster) - Appendix IV

Leal, C., Gonçalves, C., Matias, A., Rodrigues, J. and Gaspar, H. "Spice in Portugal: a source of NPS standards", *7th European Academy of Forensic Sciences Conference,* 6-11 September 2015*,* Prague, Czech Republic (Poster) - Appendix V

Gaspar, H., Ciríaco, S., **Leal, C**., Matias, A., Rodrigues, J. e Santos, S., "Tracking NPS: NMR for a rapid identification of new substances", Lisbon Addictions 2015, 23-25 September 2015, Lisbon, Portugal (Poster) - Appendix VI

Abbreviations and Symbols

^{1}H NMR – Proton Nuclear Magnetic Resonance

^{13}C NMR – Carbon Nuclear Magnetic Resonance

3,4-DMMC – (±)-1-(3,4-dimethylphenyl)-2-(methylamino)propan-1-one

4-FMC/Flephedrone – (RS)-1-(4-Fluorophenyl)-2-methylaminopropan-1-one

4F-PBP – 1-(4-fluorophenyl)- 2-(1-pyrrolidinyl)-1-butanone

4-MEC – (RS)-2-ethylamino-1-(4-methylphenyl)propan-1-one

5-MeO-DALT – *N,N*-diallyl-5-methoxytryptamine

ADHD – Attention-deficit hyperactivity disorder

AKB48 – 1-pentyl-N-tricyclo[3.3.1.13,7]dec-1-yl-1H-indazole-3-carboxamide

AM-1248 – 1-[(N-methylpiperidin-2-yl)methyl]-3-(adamant-1-oyl)indole

AM-694 - 1-[(5-fluoropentyl)-1H-indol-3-yl]-(2-iodophenyl)methanone

Buphedrone - 2-(methylamino)-1-phenylbutan-1-one

brd – Broad duplet

Caffeine - 1,3,7-Trimethylpurine-2,6-dione

Cathinone – (S)- 2-Amino-1-phenyl-1-propanone

$CDCl_3$ – Deuterated Chloroform

COSY – Correlation Spectroscopy

Council Decision – Council Decision 2005/387/JHA of 10th May 2005 on the information exchange, risk-assessment and control of new psychoactive substances

d – Duplet

Da – Daltons

dd – Double duplet

ddd – Double duplet of duplets

D_2O – Deuterium oxide

DMSO – Dimethyl sulfoxide

EI – Electron Impact Ionization

EMCDDA – European Monitoring Centre for Drugs and Drug Addiction

EMEA – European Medicines Agency

EUROPOL – European Police Office

EWS – European Union Early Warning System

FCUL – Faculdade de Ciências da Universidade de Lisboa

FFUP – Faculdade de Farmácia da Universidade do Porto

GC-MS – Gas Chromatography Mass Spectrometry

HSQC – Heteronuclear Single Quantum Coherence Spectroscopy

HMBC – Heteronuclear Multiple Bond Coherence Spectroscopy

Hz - Hertz

INCB – International Narcotics Control Board

J – Spin coupling

JWH-018 – Naphthalen-1-yl-(1-pentylindol-3-yl)methanone

JWH-122 – (4-methyl-1-naphthyl)-(1-pentylindol-3-yl)methanone

JWH-210 – 4-ethylnaphthalen-1-yl-(1-pentylindol-3-yl)methanone

JWH-250 – 2-(2-methoxyphenyl)-1-(1-pentylindol-3-yl)ethanone

LPC – Laboratório de Polícia Científica

m – Multiplet

MAM2201 – (1-(5-fluoropentyl)-1H-indol-3-yl)(4-methyl-1-naphthalenyl)-methanone

MeOD – Deuterated Methanol

MDMA – (RS)-1-(Benzo[d][1,3]dioxol-5-yl)-N-methylpropan-2-amine

MDPBP – (RS)-1-(3,4-methylenedioxyphenyl)-2-(1-pyrrolidinyl)-1-butanone

MDPV – (RS)-1-(Benzo[d][1,3]dioxol-5-yl)-2-(pyrrolidin-1-yl)pentan-1-one

Mephedrone – (RS)-2-Methylamino-1-(4-methylphenyl)propan-1-one

Methcathinone – (RS)-2-(methylamino)-1-phenyl-propan-1-one

Methedrone – (RS)-1-(4-Methoxyphenyl)-2-(methylamino)propan-1-one

Methylone – (±)-2-Methylamino-1-(3,4-methylenedioxyphenyl)propan-1-one

MS – Mass Spectra

m/z – Mass-to-charge ratio

N-ethylcathinone – (RS)-2-ethylamino-1-phenyl-propan-1-one

NMR – Nuclear Magnetic Resonance Spectroscopy

Pentedrone – (±)-1-phenyl-2-(methylamino)pentan-1-one

PMMA – 1-(4-Methoxyphenyl)-N-methyl-propan-2-amine

quint – Quintet

RMN – Espectroscopia de Ressonância Magnética Nuclear

Rt – Retention Time

s – Singlet

SWGDRUG – EI-MS Library of the Scientific Working Group for the Analysis of Seized Drugs

t – Triplet

TLC – Thin Layer Chromatography

UNODC – United Nations Office on Drugs and Crime

UR-144 – (1-pentylindol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)methanone

WHO – World Health Organisation

δ – Chemical Shift

Δ9-THC – (Δ9-tetrahydrocannabinol)

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1. Introduction

1.1. NPS – a worldwide phenomenon

According to the United Nations Office on Drugs and Crime (UNODC) [1] and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [2], based on the Council Decision 2005/387/JHA of 10th May 2005 on the information exchange, risk-assessment and control of new psychoactive substances (Council Decision) [3], a New Psychoactive Substance (NPS) is a "*substance of abuse, either in a pure form or preparation, that is not controlled by the 1961 Convention of Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat*". Therefore, when using the term NPS, one should not be biased to believe that these substances have been recently developed. On the contrary, most of these substances have been synthesised in the 1970's, some even earlier, intended for medical use; however, their chemistry has been slightly altered in order to produce similar effects to those caused by known illicit drugs [1], [4].

The phenomenon of NPS in not a new trend *per se*. What is meant is that the use of novel substances in order to circumvent existing legislation started in the 1980's with the so-called *designer drugs*, which have been defined by the International Narcotics Control Board (INCB) as "*substances that have been developed especially to avoid existing drug control measures",* referring to drugs produced from chemical precursors in clandestine laboratories or referred to as "research chemicals" [5]. The nomenclature of new substances appearing in illicit market has been evolving since then, going from "club drugs" to "legal highs" and "research chemicals" [6]. Of course, nowadays the most correct name in NPS.

There are different types of NPS. The European Police Office (Europol) and the EMCDDA have differentiated them according to their production, marketing and supply [4]. In 2012, 6 different groups of substances were defined as NPS: phenethylamines, tryptamines, piperazines, synthetic cathinones, synthetic cannabinoids and other substances that do not fall within any of the categories mentioned before [6]. However, from 2005 and up to the end of 2014, more

than 450 NPS have been reported in the European Union (EU), thus creating the need to have more classes of these substances (Figure 1). Hence, in the end of 2014, 3 more categories were considered by the EMCDDA: benzodiazepines, arylamines and opioids [7].

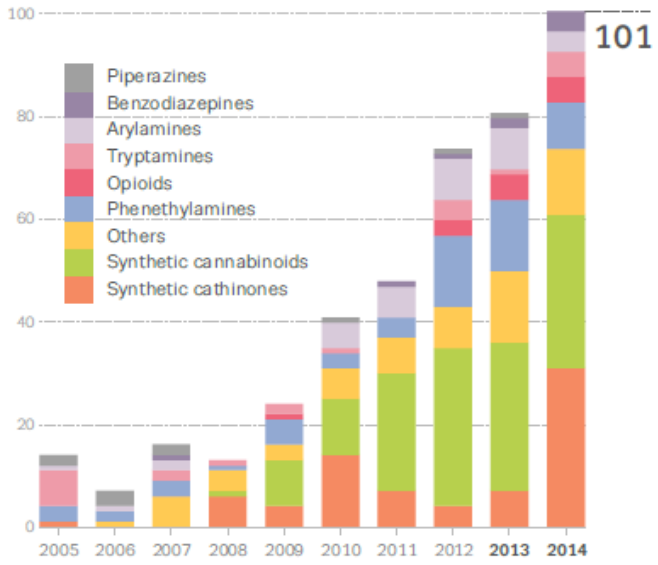


Figure 1 - NPS reported in Europe from 2005 to 2014 [7]

Also, by analysing the graphic in Figure 1, the most reported classes of NPS are synthetic cannabinoids and synthetic cathinones. Just in 2014, this two groups of substances accounted for approximately 50% of the total NPS reported in the EU. Data from 2014 reveals that, from 2008, 81 synthetic cathinones and 134 synthetic cannabinoids have been reported in Europe [8].

Reports suggest that synthetic cathinones and synthetic cannabinoids are usually sold as harmless plant feeders and herbal incenses, respectively [9, 2, 10]

1.2. EU Control of NPS and the Portuguese Legislation

At the end of the first decade of the 21st century, after a rapid emergence of NPS, governments, not only in Europe, but all over the world, where faced with the problem on how to legislate these new substances, some even considering if there was a real need to control them [11]. Having said that, governments tried to tackle the problem by creating national or regional legislation, with the goal to overcome the concerns related to the risks that these substances could present to public health [1].

Regionally, the most interesting case is the EU case. In 2005, Council Decision 2005/387/JHA was adopted in order to create a general procedure to be used by all Member States and which main focus is the assessment and rapid communication of the risks associated with NPS [1]. Basically, this is achieved in a three-step approach [2]:

a) Information exchange/ early warning
b) Risk assessment
c) Decision-making

The first approach culminates in a Joint Report by the EMCDDA and Europol that is created when it is considered that information gathered by Member States on the detection, manufacture, traffic or use of a NPS justifies a follow-up. This report is submitted to the Council of the EU, the European Commission and the European Medicines Agency (EMEA);

The Risk assessment is conducted by the scientific committee of EMCDDA and presented to the Commission and the Council when, by decision of the Council or the European Commission, it is considered that further assessment on the health and social possible problems associated with a newly identified substance is needed.

Finally, on the basis of the risk assessment, it may be decided by the Council of the EU that a new substance needs to go under control measures. These are defined by the Member States in line with their national legislation, which abide by the UN Conventions.

It should be noted that when a Member State considers it is relevant to control a substance at a national level that has not been assessed in the same way by the Council, it has the independence to do so [1], [2], [3], [4].

Up to this date, 11 joints reports (6 of which in 2014) and 7 risk assessments have been published under the terms of the Council Decision.

Across the EU, different innovative legal responses have been created in order to control the open sale of these substances [12].

Basically, they can be differentiated in three different types of response, as follows: a) Controls using consumer safety or medicines legislation (Poland

and the UK, for example); b) Extending and adapting existing laws and processes (France and Italy, for example); and c) Devising new legislation to tackle new substances (Romania and Ireland, for example) [12].

In Portugal, the approach was to create new legislation under which these NPS would be controlled, without changing already drug control legislation.

Hence, on the 17th April 2013, a new Decree-Law was published in *Diário da República*, in order to control the production, importation, exportation, advertisement, distribution, sale, possession and provision of NPS (*Dec Lei* 54/2013, of 17th April) [13]. This new decree did not bring any changes to the already existing law that controls the trafficking and possession of illicit drugs in Portugal (*Dec Lei* 15/1993, of 22nd January) [14].

1.3. Plant Feeders

Synthetic cathinones are one of the most reported classes of NPS. Just in 2014, 31 new synthetic cathinone were reported to the EWS [7]. These psychoactive compounds are usually sold covertly as plant feeders or bath salts. Plant Feeders are one of the most commonly found products in smart shops or 'head shops'. Their intended use is snorting or ingestion, although recently there have been reports of their use as injection drugs [15].

Figure 2 - Example of Plant Feeders from Portuguese Smart shops

The marketing on the packages was very smart and interesting. As seen in Figure 2, the packages were very appealing, with bright colours and suggestive names, often giving a hint on what to expect from consuming the product (for example, product named "Rush").

Another interesting fact on these packages is their back label. Figure 3 shows a commonly found label in these products. It lists ingredients and mode

of use. The ingredients list often consists of ketones and caffeine. The curious, though, is the mode of use. It is suggested that the recommended dosage should not be exceeded, unless the product is being applied on a large (i.e. adult) plant. The term large or adult may be indicative of the adult's only use of these products.

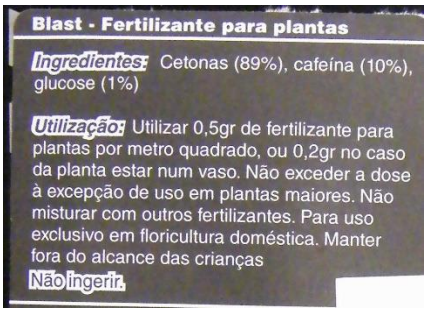


Figure 3 - Back Label of Plant Feeders

These type of products are usually presented in white powders or pills and intake modes include snorting or injection [16], which has become a serious problem in the EU and object of study by the EMCDDA [15]. Literature suggests that when plant feeders come in powdered form, they usually consist of synthetic cathinones, one of the main classes of NPS [17], [18].

Synthetic cathinones can be considered structural derivatives of the natural occurring product from the leaves of the Khat plant (*Catha edulis*), cathinone (Figure 4) [19]. The use of this plant for its stimulating effects is common practice in Eastern and Central African and Middle Eastern countries [16]. These psychoactive effects are similar to those provoked by amphetamines [20], generally behaving as central nervous system (CNS) stimulants [21].

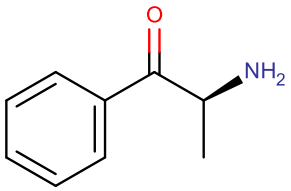


Figure 4 - Chemical Structure of Cathinone

Cathinone derivatives first appeared in the European market in the mid-2000s [22], but their synthesis has been reported since the 1920s, mainly investigated for medical purposes, but most of them have rapidly been discarded of their intended use due to severe side effects [20].

Synthetic cathinones are β-keto analogues of amphetamines, and their basic structure (Figure 5) can be altered in different ways, hence several variations can occur in the same derivative, making it possible to have dozens of different compounds considered synthetic cathinones [16], [9].

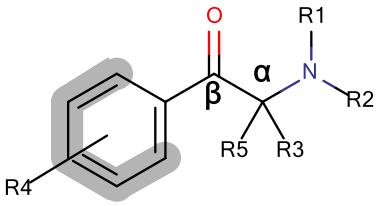


Figure 5 - General Chemical Structure of Synthetic Cathinones

For example, additional functionality may be added in the aromatic ring, either with alkyl groups or halogens (R4); different substituents at the α-carbon (R3 and/or R5), usually with an alkyl group, or *N*-alkylation (more common, but the nitrogen atom may also be part of a ring structure, mainly pyrrolidine).

Up to 2014, 81 synthetic cathinones have been reported through the EU EWS. However, just in 2014, 32 novel cathinone derivatives were reported. [7].

Cathinone is already listed in the international drug control conventions. Also, in March 2015, mephedrone, methylone and MDPV (Figure 6) were included in the schedules of the international conventions.

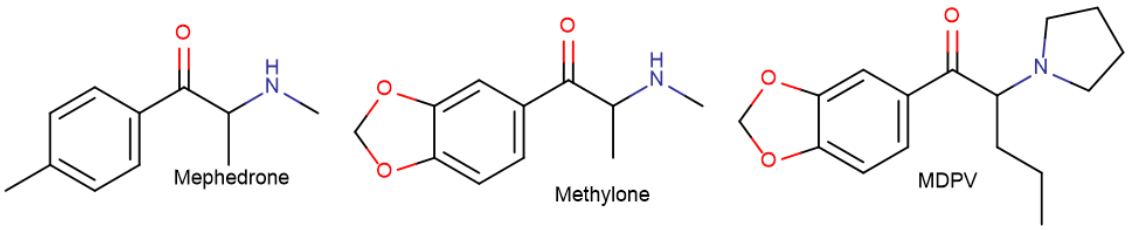


Figure 6 – Synthetic Cathinones recently added to the International Conventions

1.4. Herbal Incenses

Synthetic cannabinoids are usually sold in an herbal mixture, advertised as herbal incenses, as a smoking product. Like plant feeders, herbal incenses are one of the most widespread products sold on the 'legal highs' market. Their popularity was not immediate, being initially used only amongst experimental users. However, reports on the use of these substances as legal substitutes of cannabis and user's perspectives on online forums increased their popularity [10]. The marketing used for plant feeders was also applied to herbal incenses, making them especially appealing to young users (Figure 7).

Figure 7 - Example of Herbal Incenses from Portuguese Smart shops

These type of products started appearing in the EU market circa 2004, when herbal mixtures were being sold under the name "Spice" [10]. However, their popularity as 'legal' substituents of cannabis only reached high marks around 2008. After this increase in popularity, the first analytical data of these products was available, from different countries like Germany [23] or Japan [24]. These initial analyses revealed the presence of synthetic cannabinoids, or cannabimimetics, added to innocuous herbal mixtures. Unlike synthetic cathinones, that structurally derive from a parent compound and are all similar in structure, compounds classified as synthetic cannabinoids are so, because they are compounds with certain structural features that allow them to bind to one of the cannabinoids receptors present in the human brain, CB1 and CB2 [10], therefore mimicking the effects of classical cannabinoids, such as Δ9-THC (Δ9-tetrahydrocannabinol). Structurally, synthetic cannabinoids are very different, as seen in the comparison of Δ9-TCH and JWH-018, the first synthetic cannabinoid to be reported to the EU EWS(Figure 8).

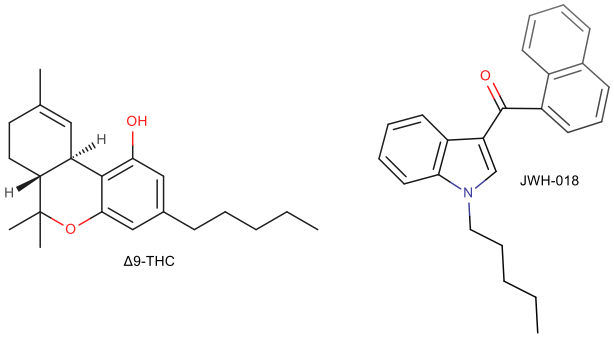


Figure 8 - Chemical structures of Δ9-THC and JWH-018

The structural features of synthetic cannabinoids allow for a generic 'structure', as seen in Figure 9.

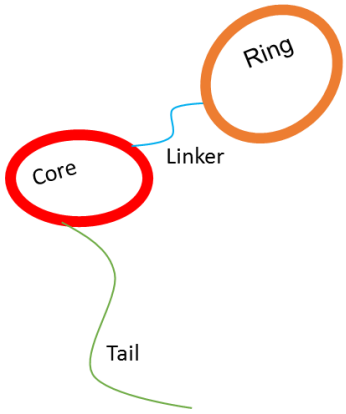


Figure 9 - Structural features of Synthetic Cannabinoids

Based on this generic structure, according to the EMCDDA, there are nowadays 14 chemical families of synthetic cannabinoids [25]. These 'families' are classified according to the core and ring structures of the compounds. Although the number of families is quite large, most reported compounds belong either to the naphthoylindole or the benzoylindole families [26]. Since the reporting of JWH-018, more 133 synthetic cannabinoids have been reported until the end of 2014. A major increase was observed in 2011, when synthetic cannabinoids more than doubled. In 2014 alone, 30 synthetic cathinones were reported to the EU EWS (Figure 10) [7], [25].

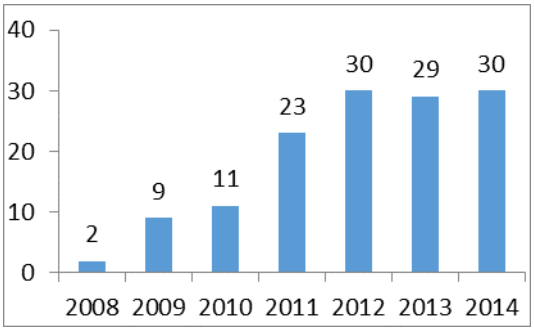


Figure 10 - Number of Synthetic Cannabinoids reported to the EWS 2009-2014

1.5. Objectives/Scope

This work was developed within the protocol established between FCUL, LPC/PJ and FFUP, following the implementation of the new legislation in Portugal, with which became necessary for the LPC to have the necessary analytical standards to analyse products that may appear within their case work.

Therefore, the goal of this project is to develop an adequate analytical methodology that allows the rapid identification of new emerging drugs of abuse from different products with different matrices, even in the absence of analytical standards. Thus, these analytical standards will be obtained either by synthesis or by purification of products from the voluntary deliveries or seized products, using Nuclear Magnetic Resonance Spectroscopy (NMR) as a tool to structurally characterise the products in order to use them as standards for other analytical techniques, such as Gas Chromatography-Mass Spectrometry (GC-MS). In the case of the herbal incenses, NMR identification is performed after isolation from the herbal matrix.

Hence, this analytical strategy is based on the premises recommended by the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUGS) in their 2014 Recommendations [27].

In their booklet, they recommend the minimum standards for the forensic identification of drugs of abuse in seized products. The use of uncorrelated techniques is advised, suggesting that when a Category A technique is used, one other technique, from any category, can used (Table 1).

Table 1 - Categories of Analytical Techniques according to the SWGDRUGS [27], page 14

Category A	Category B	Category C
Infrared Spectroscopy	Capillary Electrophoresis	Ultraviolet Spectroscopy
Mass Spectrometry	Gas Chromatography	Colour Tests
NMR Spectroscopy	Liquid Chromatography	Immunoassay
Raman Spectroscopy	Thin Layer Chromatography	Fluorescence Spectroscopy
X-ray Diffractometry	Ion Mobility Spectrometry	Melting Points

Also, as a quality control, results should be compared to data generated by a reference material, under the same analytical conditions. However, in the case of drug analysis, this is not straightforward, as reference materials are either too expensive or not readily available.

2. Experimental

2.1. Chemicals

All solvents and reagents were obtained from commercial suppliers with an analytical grade and were used with no further purification.

2.2. Equipment

GC-MS analysis performed on a gas chromatographer (Agilent® GC System 6890 Series) coupled to a mass spectrometer (Agilent® 5973 Network) with a HP-5MS column (30m × 0.25mm × 0,25µm), using ChemStation software for data acquisition and processing.

NMR spectra obtained in a Bruker Avance 400 spectrophotometer, with a frequency of 400,1MHz for ^{1}H NMR and 100,6MHz for ^{13}C NMR, using Topspin 2.1 software for data acquisition and processing. The chemical shifts were expressed as δ values and referenced to the residual solvent peak (DMSO-d_6, δH = 2.50, δC = 39.5; $CDCl_3$, δH = 7.26, δC = 77.00; methanol-d_4, δH =3.31, δC = 49.00; methanol-d_4, δH =7.16, δC =128.00) or to the signal of maleic acid (D_2O, δH = 6.42, δC = 132.16); coupling constants were reported in units of Hertz (Hz). The identifications of structures with respective assignments of the proton and carbon signals was based on the analysis of NMR spectra obtained by 1D (^{1}H, ^{13}C, APT) and 2D (including the COSY, HMBC and HSQC experiments) techniques.

2.3. Samples

Following the implementation of the new decree, all owners of any products sold in Portuguese smart shops (either consumers or retailers) were given the opportunity to voluntarily give all their products to the police, without facing any legal consequences in a 15-day period after the publication of new legislation [13].

Many stores from the Lisbon Metropolitan Area delivered that products to the police. Almost 35000 products were catalogued, according to their store (8

of them, named A to H), their type (plant feeders, herbal incenses and bath salts, for example), their brand name and their lot number.

Figure 11 shows the quantities of each type of product catalogued. From this inventory, it is possible to see that the most encountered products are herbal incenses and plant feeders. Also, plant feeders can be divided in two categories: powders and pills.

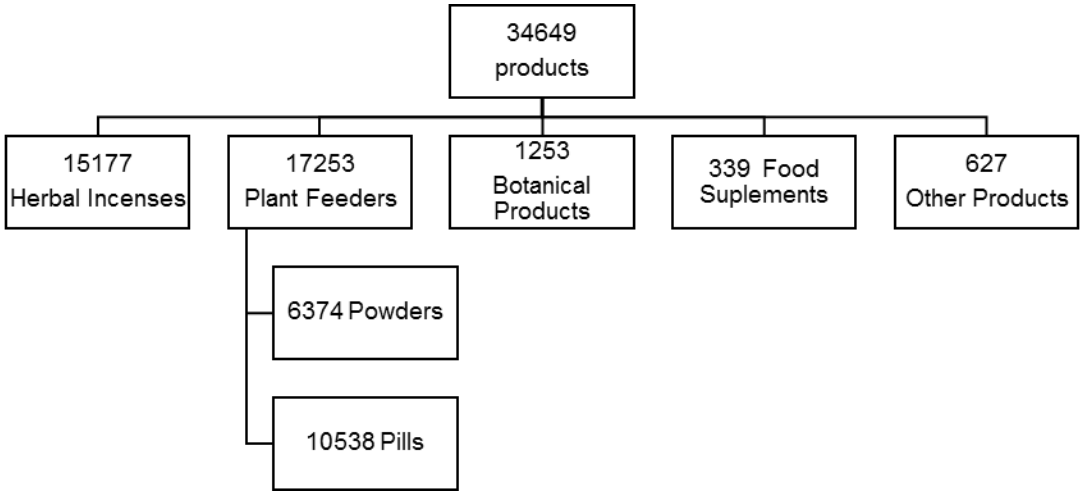


Figure 11 - Products from the voluntary deliveries

Hence, due to their large presence, this work will focus on the study of two types of products, plant feeders and herbal incenses.

Plant Feeders used as secondary standards for GC-MS analyses were acquired in Portuguese Smart shops prior to their closing.

Herbal Incenses and Plant Feeders used for variability studies were provided by the LPC and were part of the volunteer deliveries followed by the implementation of the new Portuguese legislation. This variability studies were conducted in order to assess the existence of new compounds, as to obtain as many standards as possible.

2.4. Sampling

From the 17253 plant feeders, this study will focus on the powdered plant feeders (6374 products). For the variability studies, at least 2 samples of each lot number (16 lot numbers and one ‘unknown’) of each product (Blast, Bliss,

Bloom, Blow, Charlie, Crabby, Cyclop, Darko, Demon, E.T., Kick, Mush, Rush, The Cannon, Vamp) were analysed, giving a total of 103 analysed samples.

From the 15177 herbal incenses, a first group of 33 samples was selected and analysed by GC-MS, consisting on one product of each brand name (2012, Algerian Blend, Apple, Atomic Bomb, Blow, Bombastic Kaboom Spliff Atomic Bomb, B.R.O.S., Buddah, Butterfly, Caramba, Cheese, Esfinge, Freemind, Future, Home Run, Kaboom, Magic, Mandala, MÁUI, Maya2012, Planet H, PUM!, Radioactive, Rainbow, Red Sunshine, Royal Mix, Smoke, So High, Spike99, Spliff, T-Rex, The Unicorn, Tornado, Whacked). For isolation and characterisation of synthetic cannabinoids, a second group of 23 samples was selected [Esfinge (2), Magic (2), Maya 2012 (4), Radioactive (5), Spike99 (5), Spliff (5)] for isolation and NMR analyses of the compounds.

2.5. Plant Feeders

2.5.1. Standard Solutions preparation

Methanolic solutions of the in-house standards were prepared with a concentration of 1mg/mL.

2.5.2. Sample Preparation for GC-EI-MS

Powdered samples were dissolved in methanol with a concentration of 1mg/mL

2.5.3. GC-EI-MS Conditions

Samples were injected in split mode 1:50. Helium was the carrier gas. Initial temperature set to 80 °C, held for 1 min, then increased to 270 °C at 12 °C/min and held for 7min. Injection Volume: 1µL. The injector temperature was 280 °C and the GC-MS transfer line temperature was 230ºC. Electron ionization (EI) energy was 70eV. Scan range was 35-550 *m/z*.

2.5.4. NMR Analysis

The solutions for NMR analysis were obtained from dissolved powdered samples of plant feeders (approximately 10 to 20 mg) in 500 µL of D2O.

2.6. Herbal Incenses

2.6.1. Sample Preparation for GC-MS Analyses:

Each Herbal Incense was extracted in methanol to a concentration of 100mg/mL under ultrasonication for 10/15min. The extract was filtered and 1mL placed on GC vials.

2.6.2. GC-MS Conditions:

A gas chromatographer (Agilent® GC System 6890 Series) coupled to a mass spectrometer (Agilent® 5973 Network) with a HP-5MS column (30m × 0.25mm × 0,25µm) was used. Samples were injected in splitless mode. Helium was the carrier gas. Initial temperature set to 240 °C, held for 1 min, then increased to 330 °C at 6 °C/min and held for 4min. Injection Volume: 1µL. The injector temperature was 250 °C and the GC-MS transfer line temperature was 230ºC. Electron ionization (EI) energy was 70eV. Scan range was 30-600 *m/z* [28, 2]

2.6.3. NMR Analysis

Each purified compound (10-20mg) was dissolved in 500µL of $CDCl_3$. Some compounds were also analysed in benzene (*d*6), DMSO and MeOD.

2.7. Isolation of Synthetic Cannabinoids from Herbal Incenses

2.7.1. JWH-018 from " Magic" product

Herbal product Magic (lot 2012 43X28M; smartshop H) (3g) was extracted in 100mL of methanol, under ultrasonication for 10min. Extract evaporated to dryness. Sample restituted in 4mL of chloroform and 1mL of n-hexane with silica 7734 and applied on a silica 9385 chromatographic column (18 ×4 cm) [29] packed with n-hexane. Chromatographic separation was performed by gradient elution using hexane (A) and ethyl acetate (B): 95A:5B (v/v), 30mL each for fractions 1-7; 90A:10B (v/v), 30mL each for fractions 8-33; 85A:15B (v/v), 30mL each for fractions 34-40; 80A:20B (v/v), 30mL each for fractions 41-43; 50A:50B (v/v) and 0A:100B (v/v), 200mL each, for washing of

the column. Thin Layer Chromatography (TLC) (80A:20B) permitted the separation of the fractions and the collection of JWH-018 collected from fractions 23-29 as a yellowish oil (258.1mg).

2.7.2. JWH-210 from "Esfinge" product

Herbal product Esfinge (lot 2012 43X28M; smartshop H) (3g) was extracted in 100mL of methanol, under ultrasonication for 10min. Methanolic extract evaporated to dryness. Sample restituted in 2mL of chloroform with silica 7734 and applied on a silica 9385 chromatographic column (21 ×3 cm) [29] packed with n-hexane. Chromatographic separation was performed by gradient elution using hexane (A) and ethyl acetate (B): 95A:5B (v/v), 30mL each for fractions 1-7; 90A:10B (v/v), 30mL each for fractions 8-13; 85A:15B (v/v), 30mL each for fractions 14-25; 80A:20B (v/v), 30mL each for fractions 26-32; 50A:50B (v/v) and 0A:100B (v/v), 200mL each, for washing of the column. TLC (80A:20B) permitted the separation of the fractions and the collection of JWH-210 collected from fractions 15-18 as a white powder (116.9mg).

2.7.3. JWH-122 from "Magic" product

Herbal product Magic (lot 2012 43X28M; smartshop H) (3g) was extracted in 100mL of methanol, under ultrasonication for 10min. Extract evaporated to dryness. Sample restored in 4mL of chloroform, 1mL of n-hexane and 1mL of methanol with silica 7734 and applied on a silica 9385 chromatographic column (18 ×4 cm) [29] packed with n-hexane. Chromatographic separation was performed by gradient elution using hexane (A) and ethyl acetate (B): 95A:5B (v/v), 30mL each for fractions 1-7; 90A:10B (v/v), 30mL each for fractions 8-13; 85A:15B (v/v), 30mL each for fractions 14-25; 80A:20B (v/v), 30mL each for fractions 26-32; 50A:50B (v/v) and 0A:100B (v/v), 200mL each, for washing of the column. TLC (80A:20B) permitted the separation of the fractions and the collection of JWH-122 collected from fractions 32-39, mixed with JWH-018, as a yellowish oil (188.2mg).

3. Results and Discussion

In this project, plant feeders and herbal incenses were analysed by GC-EI-MS and NMR in order to characterise the compounds present, synthetic cathinones and synthetic cannabinoids, to obtain secondary standards for routine GC-EI-MS analyses.

3.1. Synthetic Cathinones - Mass Spectra Library

The goal of this study of plant feeders is to create an in-house MS library of synthetic cathinones as to allow for the rapid identification of these group of psychoactive compounds during routine analysis. The methodology applied consists on the identification of the compounds by NMR and MS, allowing them to become secondary standards for analyses by GC-MS, as it permits a correct identification based not only on mass fragmentation, but also on different R_t for each compound.

3.1.1. Initial in-house MS library of Synthetic Cathinones

Previous work by FCUL reported the analysis of different plant feeders from Portuguese stores which allowed for the identification of different compounds, amongst which several synthetic cathinones [17]. These compounds were structurally characterised using 1D and 2D NMR spectroscopy techniques, enabling their use as analytical standards for GC-MS analyses. Therefore, 8 characterised plant feeders (Blast, Bliss, Bloom, Blow, Charlie, Crabby, Kick, Rush) were analysed by GC-EI-MS in order to add the retention times and mass spectra of 8 synthetic cathinone derivatives (3,4-DMCC, buphedrone, flephedrone, methedrone, methylone, *N*-ethylcathinone and pentedrone) (Figure 12) to build an in-house library of mass spectra of NPS (Table 2). However, some of the plant feeders analysed and used as secondary standards for GC-MS did not present only one synthetic cathinone. For example, Charlie product contained buphedrone and *N*-ethylcathinone, which, despite having similar R_t in GC, can be very well differentiated by NMR, as this technique allows the identification of the compounds, without the need to separate them or to have a purified sample.

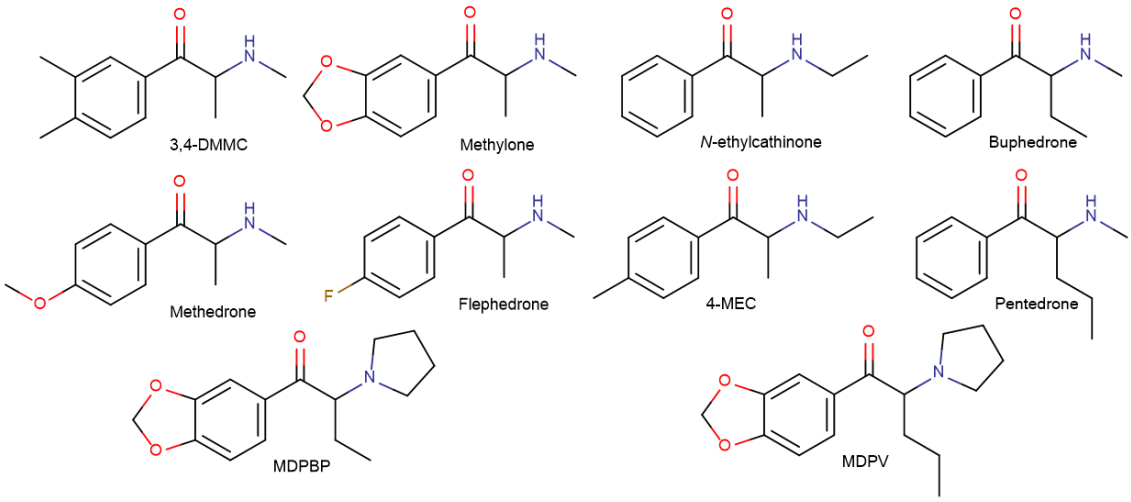


Figure 12 - Synthetic Cathinones used to create the In-house EI-MS library

Apart from the synthetic cathinones from plant feeders, two synthetic cathinones previously synthesised at FCUL were included in the library (MDPV [30] and MDPBP [31]).

Table 2 - In-house EI-MS Library of Synthetic Compounds from Plant Feeders

Compound	R_t (min)	Base Peak	Other Relevant Fragmentations
3,4-DMMC	10.55	58	191 [M^+], 133, 105, 77
4-MEC	9.81	72	191 [M^+], 119, 105, 91, 77, 65, 44, 39
Buphedrone	8.59	72	148, 105, 77, 57, 44
Flephedrone	7.60	58	166, 123, 95, 75
Methedrone	11.07	58	178, 135, 107
Methylone	12.14	58	207 [M^+], 149, 121, 42
MDPBP	15.21	112	260 [M^+], 232, 149, 121, 86, 55, 41
MDPV	15.67	126	273, 232, 149, 86, 65, 41
N-ethylcathinone	8.48	72	105, 77, 44
Pentedrone	9.55	86	105, 77, 44, 51, 39

As stated before, a unique characteristic of cathinones is the presence of a carbonyl group at the β-carbon atom, which plays an important role in the fragmentation of cathinones, due to the presence of the oxygen atom [32]. Taking the generic structure of synthetic cathinones as an example (Figure 13) and by looking at the base peaks in Table 2, it is possible to say that the side chain in the molecule of the synthetic cathinone determines its fragmentation pattern.

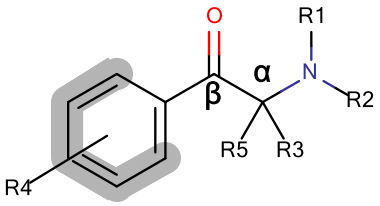


Figure 13 - Generic Chemical Structure of Synthetic Cathinones

The generic primary fragmentation of synthetic cathinones follows the pattern seen in Figure 14. The main fragmentation in cathinones (i.e. the base peak) occurs in the amino group [33]. Hence, the base peaks of the alkylamine-type cathinones are characterised by the formation of the iminium ion, which results from cleavaging of the α-carbon [32] (Figure 14), feature also common in the amphetamine class [34]. This is the reason for the low stability shown by amine molecular ions [35]. Thus, most EI-MS spectra of synthetic cathinones do not reveal the presence of the molecular ion peak.

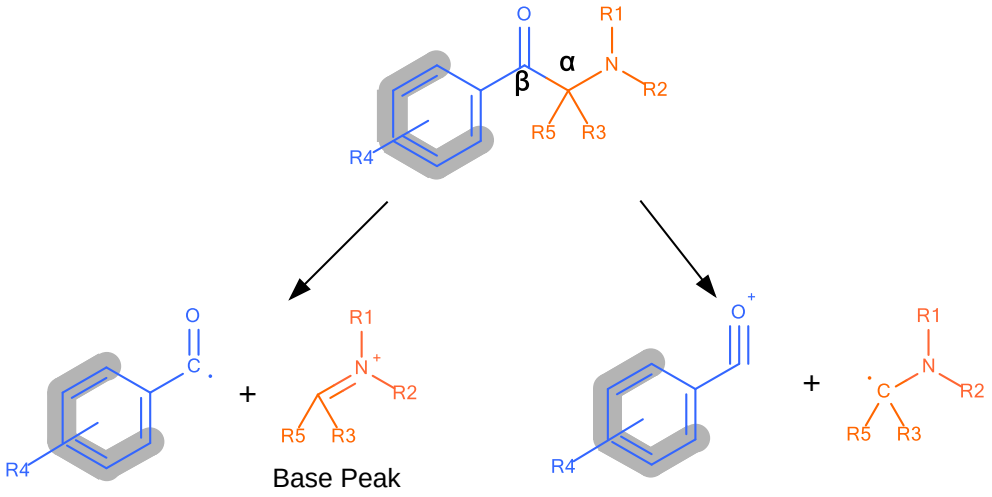


Figure 14 - Primary Fragmentation of Synthetic Cathinones

In Figure 15, the spectra of the cathinone analogues (3,4-DMMC, methedrone, flephedrone and methylone) analysed by GC-EI-MS with base peak *m/z*=58 are shown.

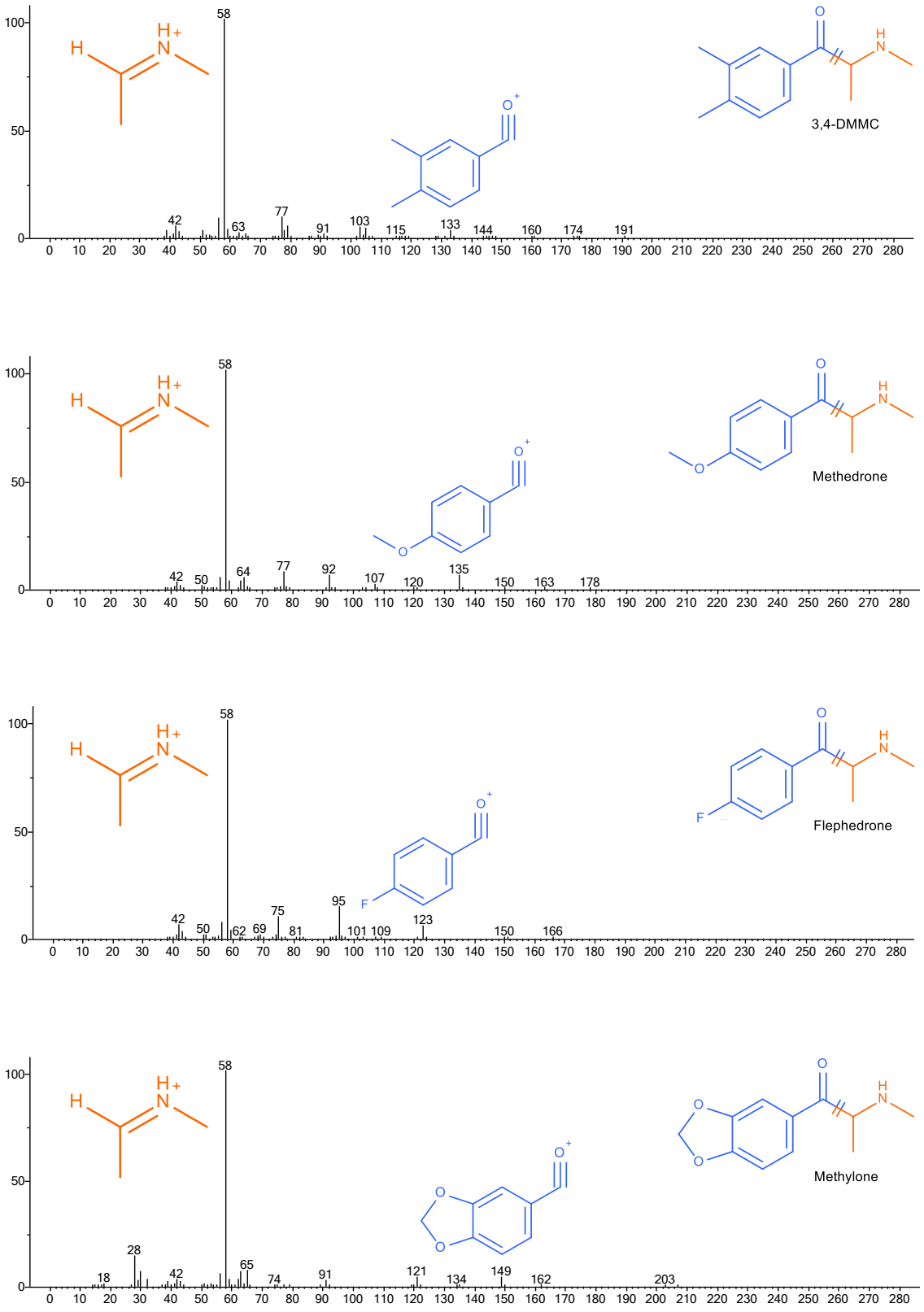


Figure 15 - EI MS Spectra of Alkylamine Synthetic Cathinones with base peak m/z=58: 3,4-DMMC; Methedrone; Flephedrone and Methylone

All of them can be considered derivatives of methcathinone (ephedrone) (Figure 16), the β-keto derivative of methamphetamine, an illicit drug of abuse [36]. The modification observed in these compounds is observed on the phenyl moiety

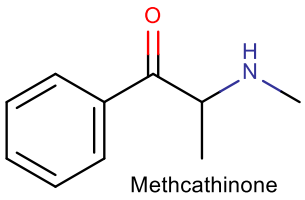


Figure 16 - Chemical Structure of Methcathinone

Hence, the described modification can clearly be detected by the fragments present in the MS if the fragments of an unsubstituted phenyl moiety are taken into consideration, as seen in Figure 17. Therefore, the characteristic fragments of the phenyl-substituted cathinones discussed next will have peaks that represent the differences in mass to the unsubstituted fragments.

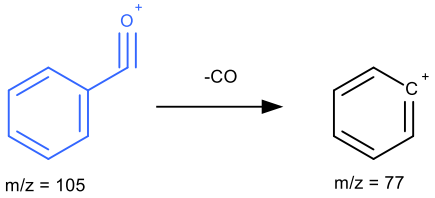


Figure 17 - Acylium and Carbenium Ions of a phenyl-unsubstituted cathinone

All of these compounds show a base peak of *m/z* 58, which is characteristic of the iminium ion ($C_3H_8N^+$) of *N*-methyl substituted methcathinones This peak results from cleavage at the bond of the α and the β carbons, with the charge being retained on the fragment containing the nitrogen atom [37]. Also worth noting is the presence of a cluster of small fragments around *m/z* 42-44 (this clutch is present in all MS in Figure 15). This has been reportedly stated as characteristic of the fragmentation of aliphatic amines, where a clutch of peaks may be found at intervals of 14 mass units [37]. Methylone, or βk-MDMA, is the β-keto analogue of MDMA (3,4-methylenedioxymethamphetamine, known as ectasy) and was patented in 1996 as an antidepressant [38]. This was of the first cathinones to be ever reported to the EWS [15]. The presence of a methylenedioxy-substitution on the aromatic

ring is clear in this compound, with the presence of two characteristic peaks at *m/z* 149 ($C_8H_5O_3^+$) and *m/z* 121 ($C_7H_5O_2^{+)}$, indicating the presence of the methylenedioxybenzoyl and methylenedioxyphenyl cations, respectively. The latter results from the loss of CO (28 Da) from the first fragment (Figure 18).

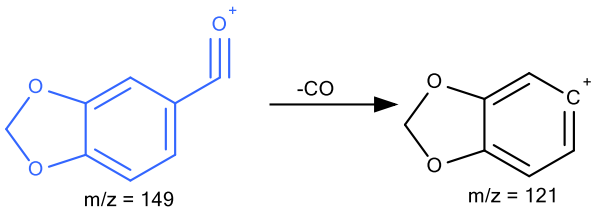


Figure 18 - Secondary Fragmentation on the Acylium Ion of Methylone

These two fragments have also been reported for other compounds with this substitution. However, they have also been reported in the case of positional isomers, where the methylenedioxy group is in the 2,3 position [39]. Hence, NMR is used in order to elucidate the structure of the compound [38].

Flephedrone (4-FMC or 4-fluoromethcathinone), is a methcathinone with a fluor substitution on the aromatic ring. This compound was first synthesised in 1952 in order to explore its attributes as antihyroidal and antibacterial compound. However, according to the World Health Organisation (WHO), there is no medical use reported [40]. As seen in the case of methylone, its substitution on the aromatic ring allows the identification of fluor-substituted cathinones.

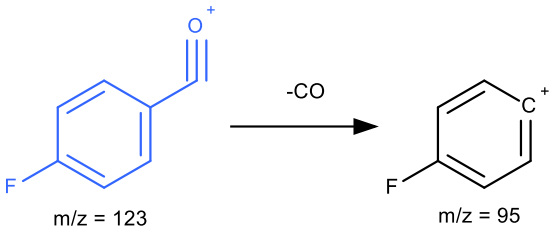


Figure 19 - Secondary Fragmentation on the Acylium Ion of Flephedrone

In Figure 19, the two characteristic fragments of a fluor substitution are shown. Firstly, the acylium ion at *m/z* 123 ($C_7H_4OF^+$), and then the fragment formed after the loss of a carbon monoxide, *m/z* 95 ($C_6H_4F^+$). These two fragments, correspondent to the fluorobenzyloxy and fluorophenyl cations, respectively, are present in MS of flephedrone, shown in Figure 15. It should be

noted that the position of the fluor atom cannot be determined by the analysis of this EI-MS fragments. Archer reported the identification of 3-fluoromethcathinone, after identifying it uncorrectly as being flephedrone, merely based on GC-MS analysis [41]. However, in this case, the presence of flephedrone, in which the fluor atom is present in position 4, has been confirmed by NMR. Methedrone and 3,4-DMMC are the other two synthetic cathinones present in Figure 15 with the same base peak (*m/z* 58). Hence, following the premise of the fragment produced in the aromatic region by cleavage on the β bond to the ring in aryl alkyl ketones, 3,4-DMMC (3,4-dimethylmethcathinone) and methedrone have characteristic acylium fragments at *m/z* 133 ($C_9H_9O^+$) and 135 ($C_8H_7O_2^+$), respectively (Figure 20).

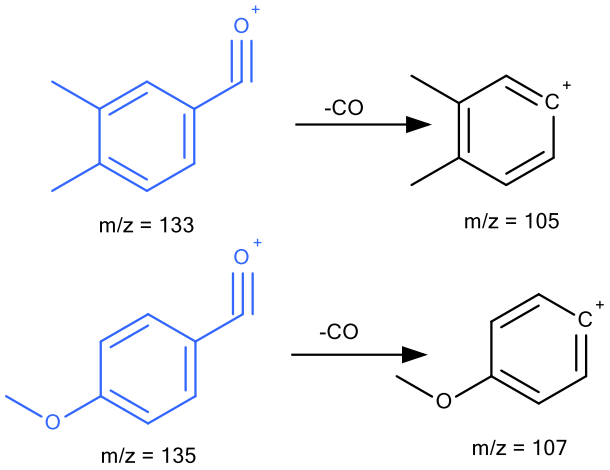


Figure 20 - Secondary Fragmentation on the Acylium Ions of 3,4-DMMC and Methedrone

3,4-DMMC has not been much reported. In fact, cathinones (and even the amphetamine analogues) with a 3-position substitution were not much discussed up to 2012 [42]. This compound has a 3,4-dimethyl substitution on the aromatic ring, hence its fragment [105+28 (two methyl groups minus 2 hydrogen atoms)] [43] and methedrone has a methyloxy substitution, having a fragment at 135 [105+30 (methyloxy group minus one hydrogen atom)] (Figure 17). Methedrone shows a rather high toxicity, as many of the methoxylated synthetic cathinones [18]. It is the β-keto analogue of paramethoxymethamphetamine (PMMA), being responsible for two fatalities, up to 2010 [44]. Those acylium ions undergo further fragmentation, forming characteristic fragments with lower intensities *m/z* 105 ($C_8H_9^+$) and *m/z* 107 ($C_7H_7O^+$), respectively for 3,4-DMMC and methedrone [18], [32] (Figure 20).

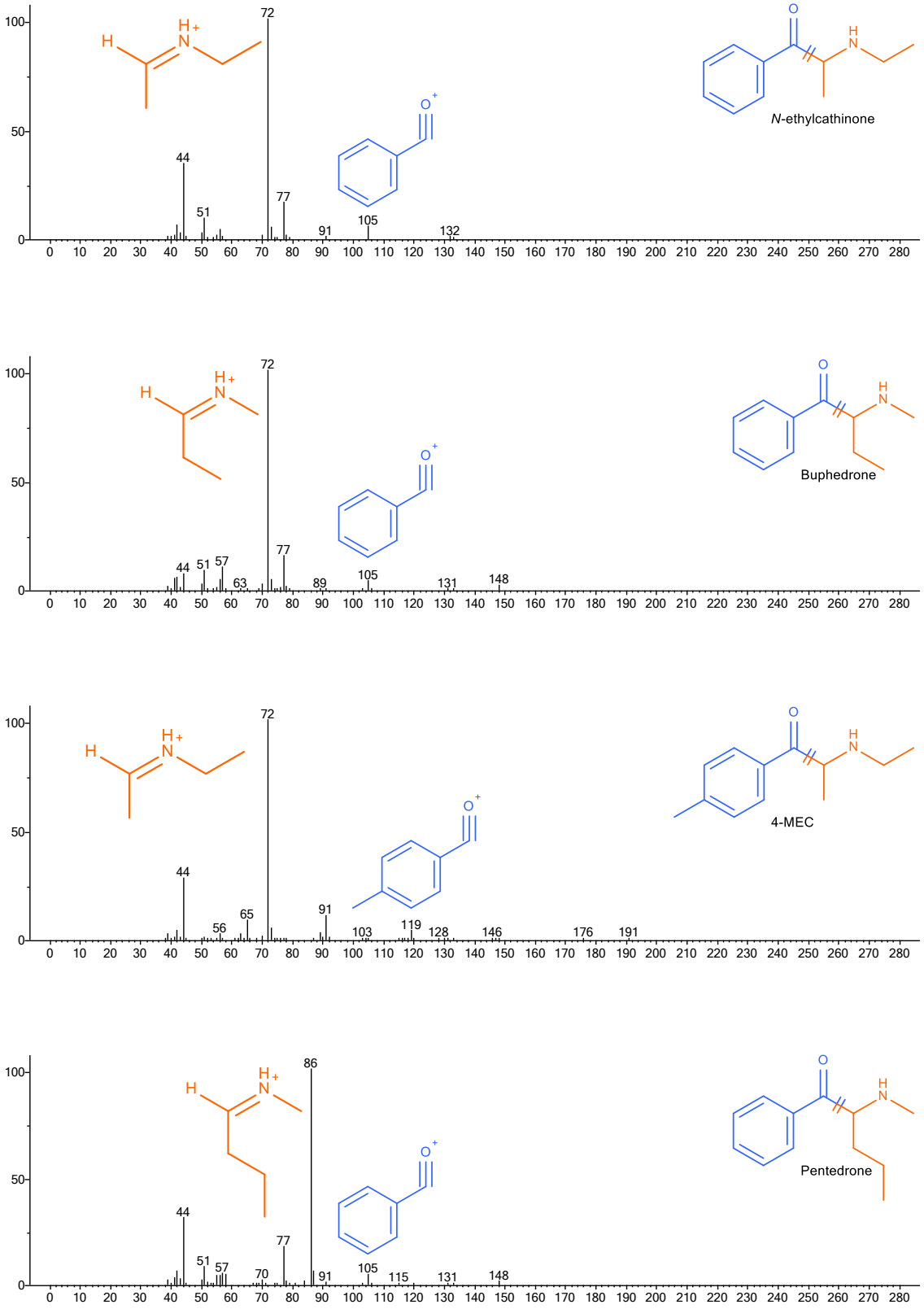


Figure 21 - EI MS Spectra of Alkylamine Synthetic Cathinones with base peak m/z=72 (N-ethylcathinone; Buphedrone; 4-MEC) and with m/z=86 (Pentedrone).

In Figure 21, the spectra of the cathinone analogue sanalysed by GC-EI-MS with base peak *m/z* 72 ((4-MEC, *N*-ethylcathinone, buphedrone) and *m/z* 86 (pentedrone) are shown. All three cathinone derivatives with a base peak of 72Da show similar spectrometric behaviour and hence, are structurally similar. This base peak has been reported as one of the most difficult one to distinguish between cathinones, as it is present in six commonly occurring compounds (4-MEC, *N*-ethylcathinone, buphedrone, ethylone, butylone and DMMC) [32].The most distinguinshable one in this case is 4-MEC, due to its methyl substitution on the aryl moiety (hence employing the aminium fragmentation).

Due to being structurally very similar, the spectrometric behaviour of buphedrone and *N*-ethylcathinone is, at first glance, the same. One could assume that being different compounds, they would show different chromatographic behaviour. However, as seen in Figure 22, their retention times differ only by a decimal place, which is not enough for a satisfactory differentiation.

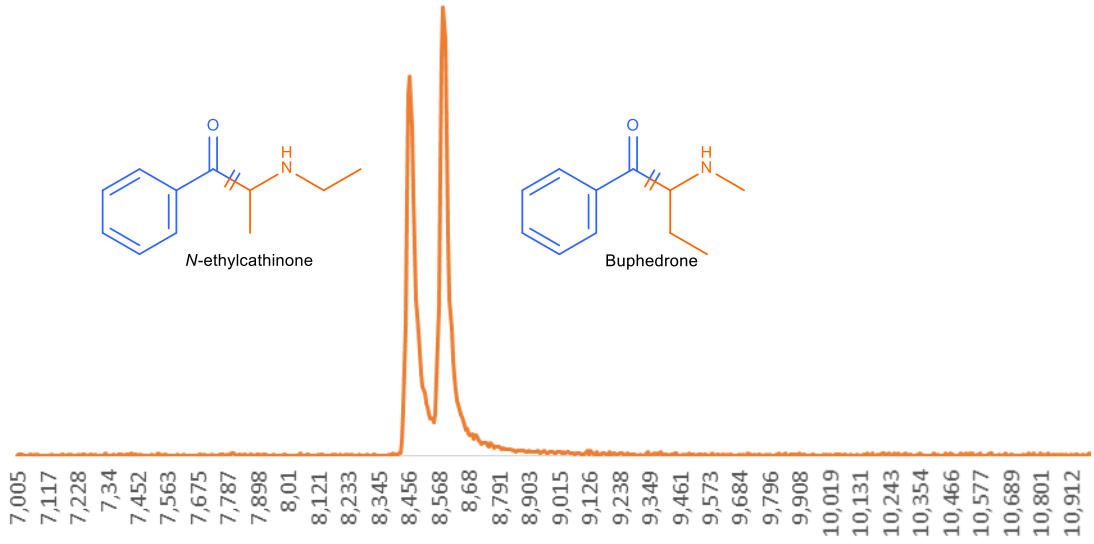


Figure 22 - Chromatographic Separation of Secondary Standards of N-*ethylcathinone and Buphedrone*

The only structural difference between these two cathinone derivatives is the ethyl substitution. Buphedrone is an α-ethyl substituted cathinone, with literature references of its synthesis and initial use as a medicine for diabetes from 1928 [45], while *N*-ethylcathinone has its ethyl-substituion on the nitrogen atom. Based on the fact that the primary fragmentations of synthetic cathinones occur by cleavage of the α and β carbons, they are not sufficient to tell apart

this two compounds. It is therefore necessary to take a closer look at secondary fragmentations of these two positional isomers.

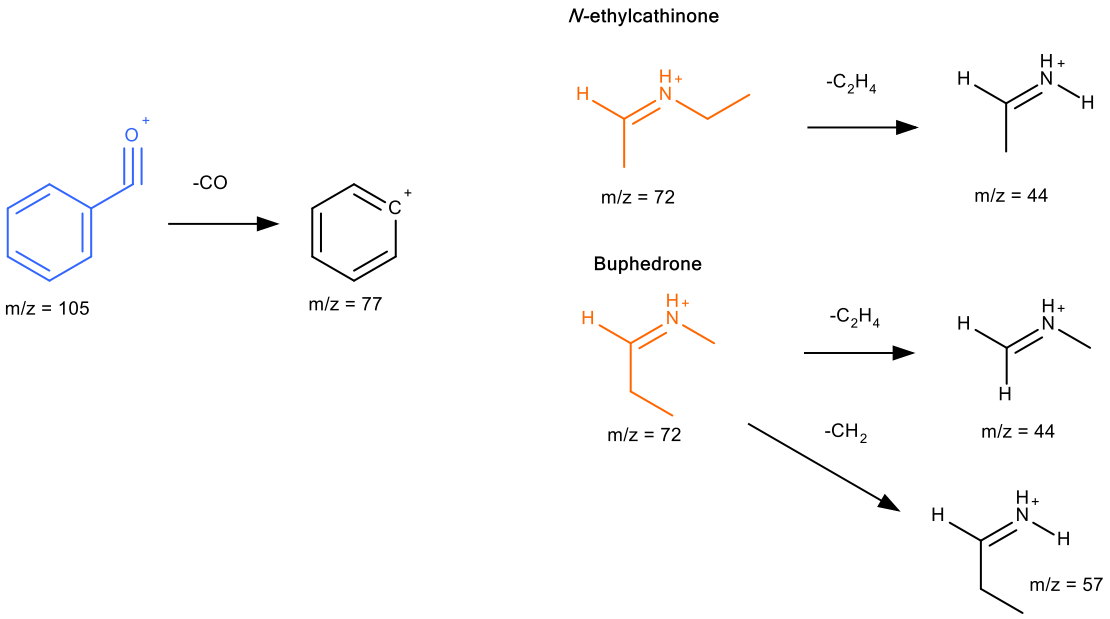


Figure 23 - Secondary Fragmentations on the Acylium and Iminium Ions of N-ethylcathinone and Buphedrone

As seen in Figure 23, the secondary fragmentations of *N*-ethylcathinone and buphedrone are not very helpful in the structural determination of these compounds. The acylium ion is the same [*m/z* 105 ($C_7H_5O^+$)], thus they show the same carbenium ion after the loss of a CO group [*m/z* 77 ($C_6H_5^+$)]. And structurally, their iminium ions are different, but their *m/z* values are the same [*m/z* 72 ($C_4H_{10}N^+$)] and hence the value of the secondary fragmentation of this ion is also the same [*m/z* 44 ($C_2H_6N^+$)]. However, the behaviour of the secondary fragmentation of the iminium ions is different. The general fragmentation of synthetic cathinones suggests the loss of neutral species from the iminium ion, leading to consequent lower mass iminium ions. This secondary fragmentation results largely from the loss of alkene groups, conceding ions of great relevance for structural elucidation [35]. In order for this consequent fragmentation to occur, at least an ethyl moiety substitution must be present. This substituent group is split from the heteroatom with hidrogen transfer from the leaving group to the latter [35].

In the study of these two compounds, only *N*-ethylcathinone has an ethyl-substituion on the nitrogen atom; of course, buphedrone also has an ethyl substitution, but on the α-carbon. This secondary fragmentation is therefore

more plausible to occur in the case of *N*-ethylcathinone, as seen in the MS in Figure 21, because nitrogen has stronger charge-stabilizing properties than carbon [35].

Although not much abundant, this phenomenon also occurs in buphedrone, with the loss of the methyl-substitution on the nitrogen atom, leading to fragment *m/z* 57 ($C_3H_8N^+$), usually more abundant than fragment *m/z* 44. This fragment is in accordance to previous reported analyses of this compound [46], [47] and is not present in the MS of *N*-ethylcathinone.

Nevertheless, the secondary fragmentation of the iminium ion of these two cathinone derivatives [intense peak at *m/z* 44 ($C_2H_6N^+$) for *N*-ethylcathinone and peaks *m/z* 44 ($C_2H_6N^+$) and *m/z* 57 ($C_3H_8N^+$) for buphedrone] allows for their spectrometric differentiation and could be an important tool on the analysis of new substances that may emerge. However, these two compounds were added to the in-house library from the same product, a Charlie plant feeder. Although their differentiation by GC-MS is rather complicated, NMR allows a clear distinction between this two positional isomers, even if they are analysed as a mixture.

4-MEC (4-methylethcathinone) shows the same fragmentation pattern as *N*-ethylcathinone in the formation of the iminium ion and subsquent sub-fragmentations, showing a relatively abundant peak at *m/z* 44, characteristic of the loss of the alkene group from the nitrogen atom. As expected in the case of cathinone derivatives, this fragmentation also leads to the formation of an acylium ion. In spite of that, it shows different fragments from *N*-ethylcathinone or buphedrone, yet characteristic of a methyl substitution on the aromatic ring.

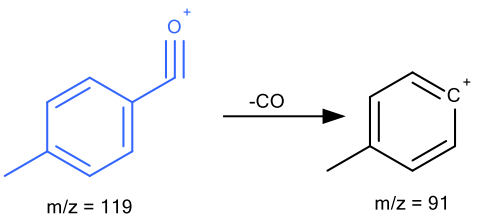


Figure 24 - Secondary Fragmentation of the Acylium Ion of 4-MEC

The peak at *m/z* 91 ($C_7H_7^+$), the tropylium ion [32] (Figure 24), is generally obtained from fragmentation processes of phenylalkanes and is

therefore an important peak, as it is present in most spectra that contain phenylalkane structures [35].

Further fragmentation of the tropylium ion can also be rather useful when aiming at understanding the presence of an alkyl-substitution on the aromatic ring. Aromatic-unsubstituted cathinones reveal the presence of low abundant ions at *m/z* 51 ($C_4H_3^+$) and *m/z* 39 ($C_3H_3^+$), result from the loss of an acetylene group and an allene diradical, respectively. Although helpful in this determination, as stated before, these fragments are not much abundant, as the benzyl core structure is rather stable [48]. Despite that, only fragment *m/z* 39 is observed in the case of 4-MEC. Instead of *m/z* 51, fragment *m/z* 65 ($C_5H_5^+$) is observed (Figure 25).

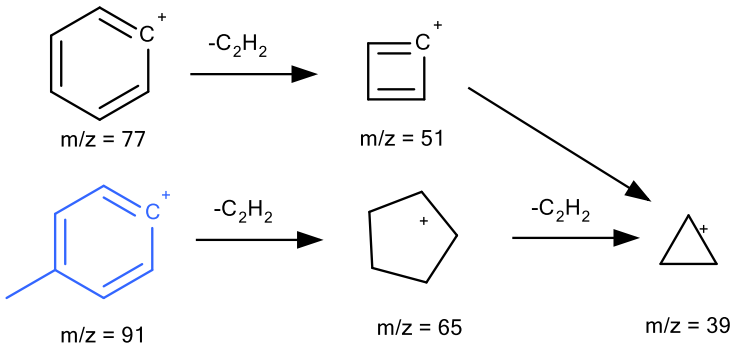


Figure 25 - Fragmentations resulting from the loss of small molecules from Aromatic Ions

The last compound from Figure 21 is pentedrone with a base peak of *m/z* 86 ($C_5H_{12}N^+$), an acylium ion (*m/z* 105), carbenium ion (*m/z* 77) and peaks 51 and 39, typical of a cathinone derivative unsubstituted in the aromatic ring. Alike buphedrone, references of its use in the late 1920s can be found in the literature [45].

Although referred as one of the possible iminium ions in the fragmentation of synthetic cathinones, peak at *m/z* 86 is not that common. In this study, for example, pentedrone is the only cathinone derivative showing 86 as its base peak. Literature is in accordance with the observed in this study. In his study on the fragmentation of different NSP, Zuba only describes two cathinones with a base peak at 86, them being pentedrone and pentylone, the benzyl methylenedioxy substituted analogue of pentedrone [32]. In fact, these

two compounds are often found on the same studies, specially when fragmentation patterns on EI-MS are being discussed [49].

As observed for buphedrone, in pentedrone the secondary fragmentation does not follow the general loss of the alkyl chain in the nitrogen atom, as it only has an *N*-methyl substitution. Having said that, and as seen in Figure 26, fragmentation occurs on the propyl substitution on the α-carbon. This fragmentation is suggested by Westphal et al in their study on the characterisation of pentedrone [49]. Although not on the nitrogen atom, the process by which the iminium ion fragments is also characteristic of aliphatic amines and is, in principle, a second α-cleavage, confirming the loss of the propyl substituent [35].

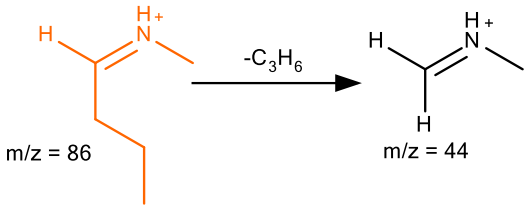


Figure 26 - Secondary Fragmentation of the Iminium Ion of Pentedrone

It has been suggested in literature that, as it has been reported for cathinone, synthetic cathinones may undergo processes of cyclisation and rearrangement to an isomeric product [21]. This reorganisation could be achieved via a dihydropyrazine dimer [50].

In the publication on the analysis of plant feeders by our group in collaboration with FFUL, the presence of the iso-product of pentedrone, isopentedrone, was found [17]. According to that study, a content of 11% of isopentedrone in a mixture with pentedrone was found [17]. Hence, the secondary standard of pentedrone used also revealed the presence of isopentedrone. As seen in Figure 27, the presence of isopentedrone can also be verified in GC analyses.

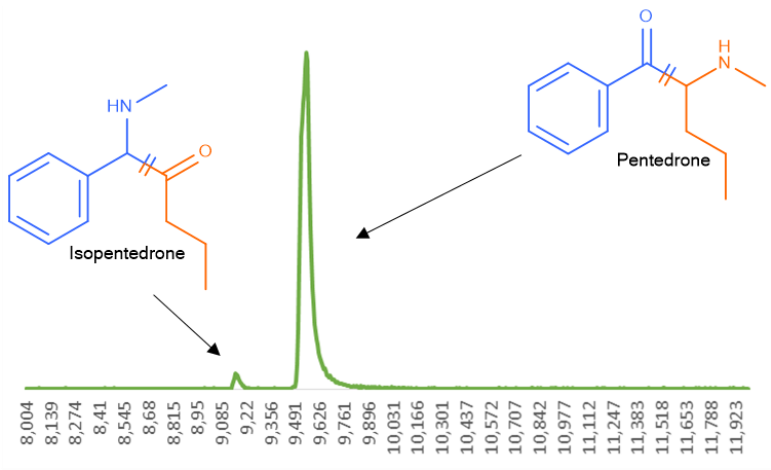


Figure 27 - Chromatographic Separation of Pentedrone and its by-product, Isopentedrone

Table 3 - GC-EI-MS Characteristics of Isopentedrone

Compound	R_t (min)	Base Peak	Other Relevant Fragmentations
Isopentedrone	9.162	120	104, 91, 77, 42

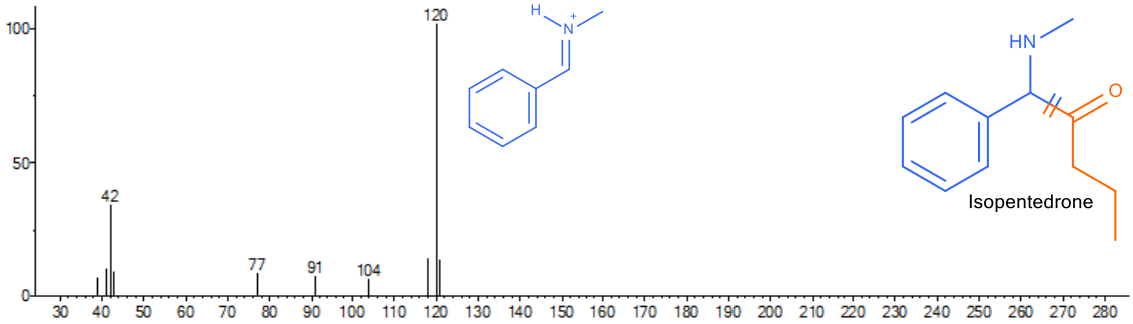


Figure 28 - EI-MS of Isopentedrone

The main structural difference between pentedrone and isopentedrone is the location of the amino and the keto moieties, which essentially changed their place in the molecule. This shift in the chemical structure leads to a completely different MS (Figure 28), although the spectrometric behaviour is similar (Figure 27 and Table 3) [49].

MS of isopentedrone showns an intense peak, the base peak, at *m/z* 120 ($C_8H_{10}N^+$). This results, as expected, from α-cleavage leading to the formation of a *N*-methylbenzyliminium ion.

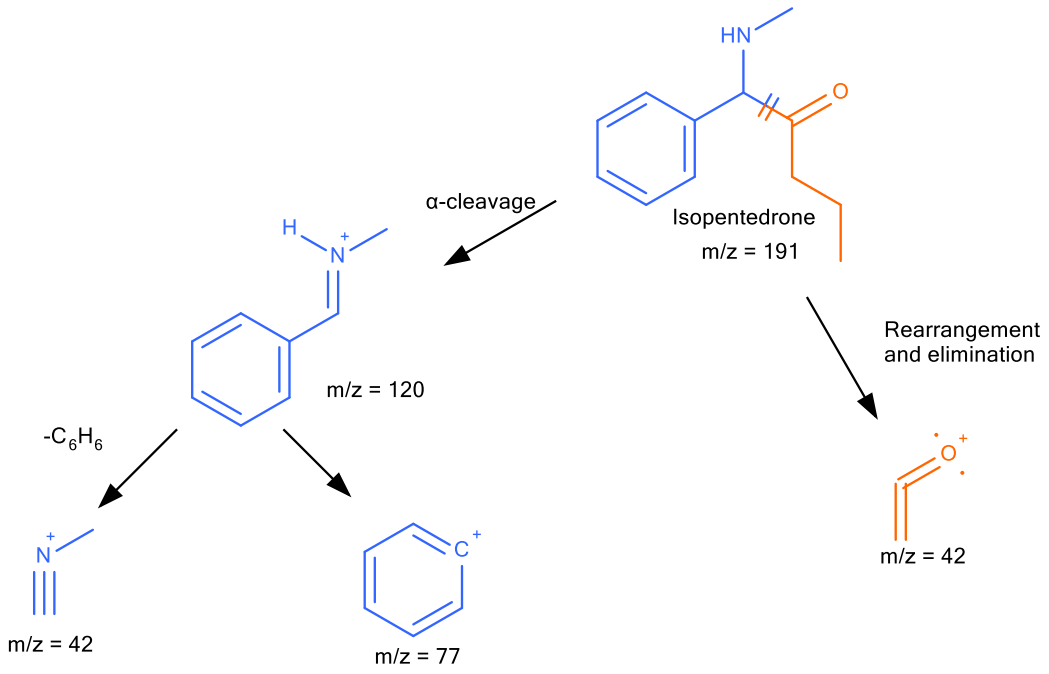


Figure 29 - Primary and Secondary Fragmentations of Isopentedrone

Further fragmentation of the iminim ion can lead to the formation of a carbenium ion [*m/z* 77 ($C_6H_5^+$)] or to the appearance of a peak at *m/z* 42 ($C_2H_4N^+$), which is formed by elimination of the benzene group from the iminium ion. However, another possible conformation for this peak at *m/z* 42 is a rearrangement and elimination process on the keto side of the chain ($C_2H_2O^+$) (Figure 29). This proposition, given by Westphal et al in their study on the analysis of isocathinones by-products [49], suggests a rearrangement on the keto chain, followed by elimination of ethene and methylbenzylamine, resulting in the formation of a ketene radicalcation. This suggested rearrangement, succeeded by the loss of an alkene, is known as the McLafferty Rearrangement, which is based on the transfer of a γ-hydrogen atom with cleavage of the β-carbon to the carbonyl site. This can only happen if at least one γ-hydrogen is available that could cause the loss of an alkene group after β-cleavage [35]. In other words, this rearrangement could occur if one alkyl group with at least three carbons in length is attached to the carbonyl group of a ketone [51].

The remaining two synthetic cathinones present in the EI-MS library are compounds that, on the contrary to those studied so far, belong to the pyrrolidine-type cathinones. They are MDPV and MDPBP, and as mentioned

before, these two standards were not acquired from the products of portuguese smart shops, but were synthesised by our group at FCUL [30, 31].

These two compounds are considered a derivative on another compound, pyrovalerone [(RS)-1-(4-methylphenyl)-2-(1-pyrrolidinyl)pentan-1-one], a synthetic compound developed as a medicine in the 1960's, but that was withdrawn to the high dependence effects (Figure 30).

The difference between pyrovalerone and MDPV and MDPBP lies on the methylenedioxy substitution on the aromatic ring instead of a methyl group in the case of pyrovalerone (MDPBP also differs in the alkyl substitution on the α-carbon).

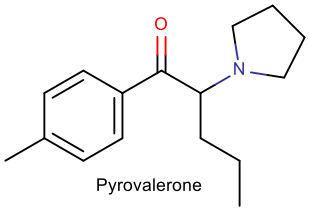


Figure 30 - Chemical Structure of Pyrovalerone

According to the EMCDDA, MDPBP was first reported in the EU in March 2010 by the United Kingdom [15]. However, when publishing the structural characterisation of this compound in 2011, Westphal et al refer the seizure of a white powder containing this product in February 2009 [52].

MDPV was first identified in Germany in 2007 [53] and its occurrence in Europe hit the maximum after mephedrone, another synthetic cathinone, began to be controlled in several countries [54]. Hence, MPDV could be considered the "legal" substitute od mephedrone back when it first appeared.

However, and although all these substances are currently controlled in Portugal, MDPV plays an important role, as it has gone through the three-step process to control NPS in the EU. On January 2014, the EMCDDA and Europol published a Joint Report on MDPV, based on information gathered from a procedure initiated on October 2013 for the collection of information on this synthetic cathinone. Such information was mustered primarily through the Reitox National Focal Points. After decision of the Council, a risk assessment was conducted by the two european agencies and published on May 2014. This

risk assessment was then analysed by the Council, who, on the 25th September 2014, published Council Implementing Decision 2014/688/EU of 25 of September on subjecting 25I-NBOM, AH-7921, MDPV and methoxetamine to control measures [55]. The merits of the basis of controlling MDPV include 108 fatalities and 525 non-fatal intoxications in different Member States. On Article 2, it is stated that the Member states have to place these substances in the appropriate legislation in order to comply with the 1971 UN Convention on Psychotropic Substances. In Portugal, this means that MDPV, currently scheduled under *Decreto-Lei 54/2013* [13], shall be moved to an amendment of *Decreto-Lei 15/93*, of 22nd January, relative to the legal framework applicable to the trafficking and consumption of narcotics and psychotropic substances [14].

Although not alkylamine-type cathinones, MDPV and MDPBP are still structurally similar to any other synthetic cathinone and hence, their spectrometric behaviour is expected to be conformable to what is generally observable (Figure 31).

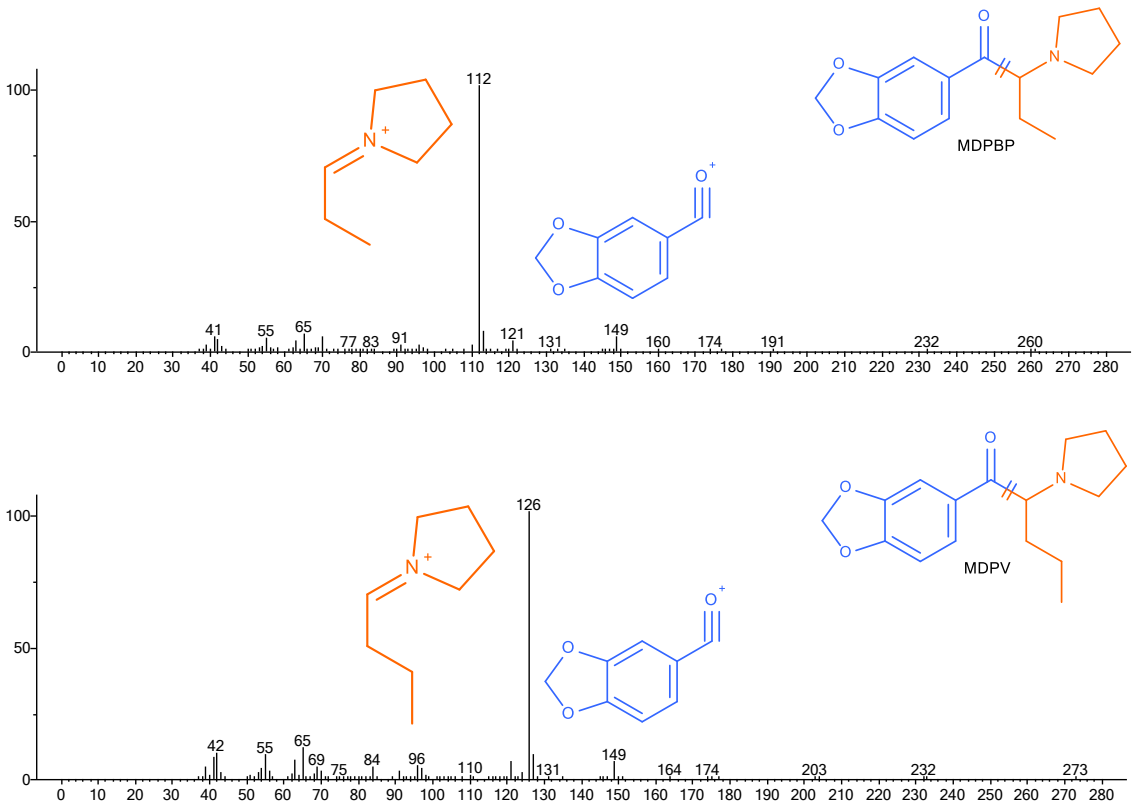


Figure 31 – EI MS Spectra of Pyrrolidine Synthetic Cathinones with base peak m/z=112: MDPBP and m/z=126: MDPV

After α-cleavage, the formation of an acylium ion at *m/z* 149 occurs in these two compounds, suggesting the presence of an aromatic moiety, substituted with a methylenedioxy group, as seen for methylone in Figure 18 and therefore not further discussed.

On the other hand, their iminum ions and subsequent fragmentations are rather characteristic. The presence of a pyrrolidine group on one of the sides of the carbonyl group creates interesting and rather specific fragmentations for these compounds. As expected, the iminium ion in pyrrolinide-type cathinones follows the same formation pathway as for the alkylamines types, being formed after α-cleavage of the carbonyl group, and creating an abundant base peak.

As seen in the obtained MS for these compounds (Figure 31), MDPBP and MDPV have base peaks of *m/z* 112 ($C_7H_{14}N^+$) and *m/z* 126 ($C_8H_{16}N^+$), respectively. The difference of 14Da between them can be explained by the alkyl substitution on the α-carbon, which is an ethyl group for MDPBP and a propyl group for MDPV. Westphal et al suggest another minor, but possible, α-cleavage, leading to the formation of ion *m/z* 232 ($C_{13}H_{14}O_3N^+$) in both compounds. This is observable in the presented EI-MS for the two compounds and, although very low abundant, it represents the loss of the alkyl chain on the α-carbon, leaving a fragment characteristic of a methylenedioxy substituted pyrrolinide-type cathinone (Figure 32).

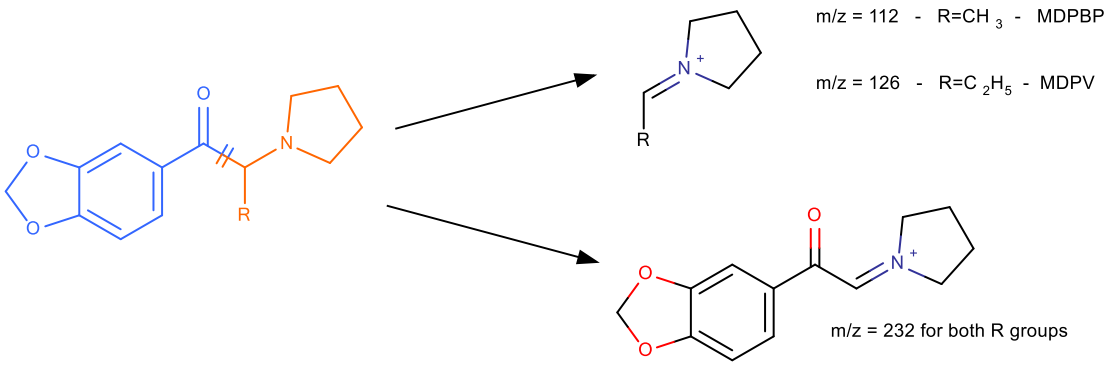


Figure 32 - Possible α-cleavages for pyrrolidine-type cathinones, as proposed by Westphal et al [56]

Following the primary fragmentations, the abundant α-cleavage undergoes further fragementations on the pyrrole ring. These fragments are present at the low mass region of the spectra, which could be complicated to

attribute, as they may not be very specific. Regardless, Zuba and Westphal et al suggest several secondary fragments characteristic of any synthetic cathinone with a pyrrolidine ring on the side chain: *m/z* 86 ($C_5H_{11}N^+$), *m/z* 70 ($C_4H_8N^+$) (or *m/z* 69) *m/z* 55 ($C_4H_7^+$), *m/z* 42 ($C_3H_6^+$) and *m/z* 41 ($C_3H_5^+$) [32], [57] (Figure 33).

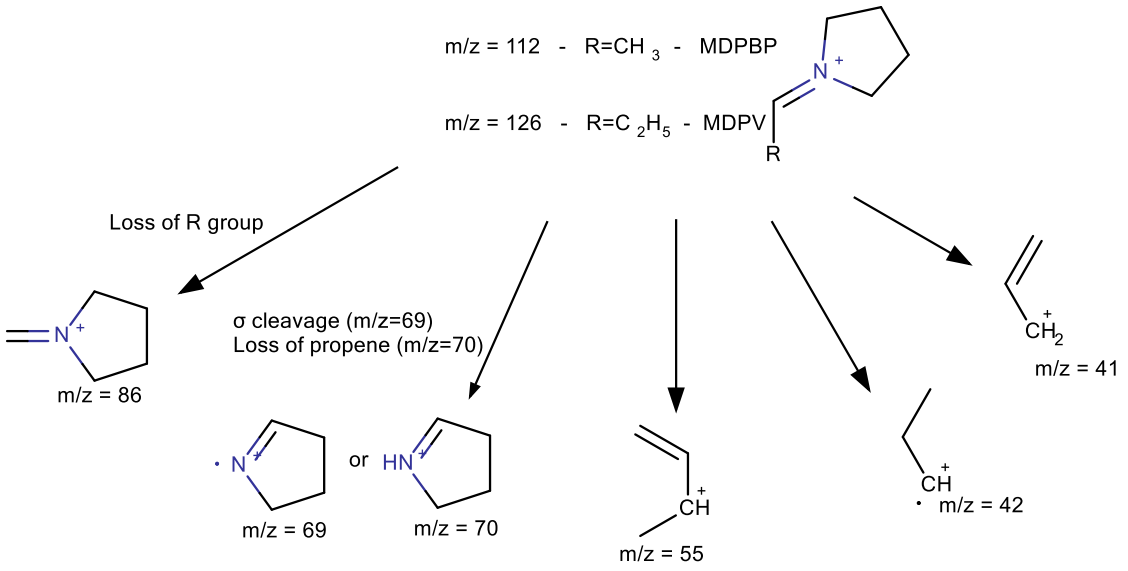


Figure 33 - Secondary fragmentations on pyrrolidinophenones imminum ion

These suggested fragments are seen in the EI-MS spectra of the secondary standards analysed.

An intriguing peak usually appears on the GC chromatogram of pyrrolidinophenone structures, even in the case of a pure compound (Figure 34). This peak may be present as a GC artefact, although authors have suggested that this could be a synthesis by-product [52]. However, its presence is only noticeable on GC, making it more plausible to be an artefact.

The molecular weight of this artefact [*m/z* 259 ($C_{15}H_{17}O_3N^+$) and the base peak at *m/z* 110 ($C_7H_{12}N^+$), suggest a dehydrogenation on the pyrrolidine moiety [52]. The presence of this peak could be of interest, as its presence has been reported on GC analyses of other pyrrolidinophenones [52] (Figure 35).

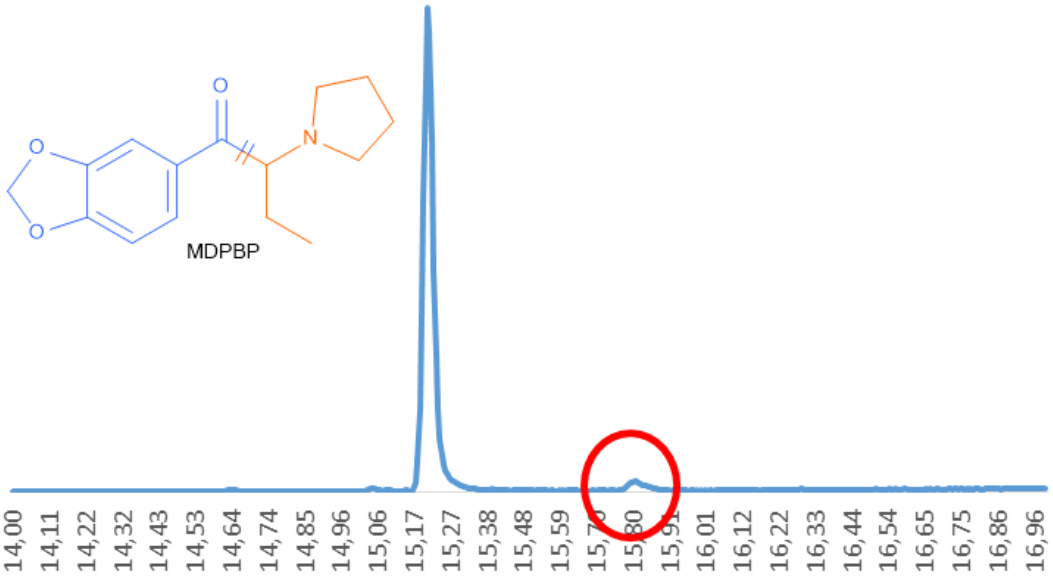


Figure 34 - Chromatogram of MDPBP Standard with the presence of an artefact (highlighted in red)

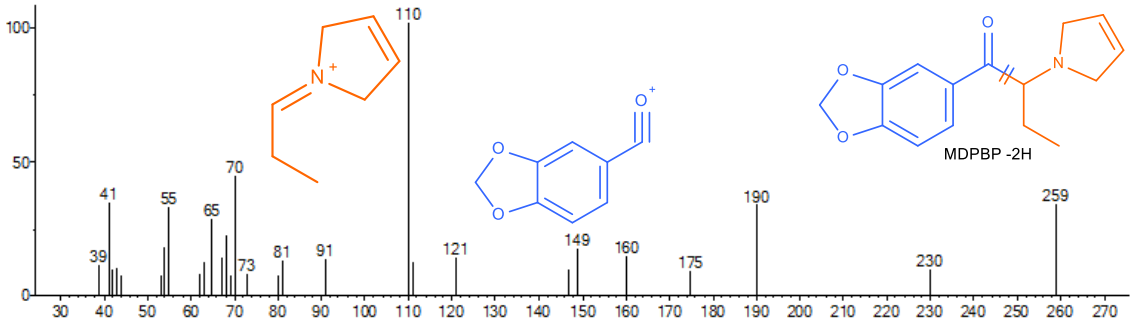


Figure 35 - EI-MS of MDPBP -2H, artefact present in GC analysis

As seen in the previous pages, synthetic cathinones follow a general fragmentation pattern, making it possible to group different characteristic fragments in order to facilitate the analysis in a forensic laboratory. The construction of this database allows the addiction of R_t to each compound, making it a valid tool in NPS identification. The identification of synthetic cathinones by GC-MS using these secondary standards allows the analysis of new samples, as it gathers information from both techniques. For each compound, a R_t and a MS are obtained from GC and MS, respectively.

3.1.2. 4F-PBP: Identification of a novel NPS

The EI-MS in-house library has allowed for the identification of the compounds present in casework samples from LPC. In January 2015, 3 white powder samples were analysed by GC-MS, showing one major peak in the chromatogram and, initially, entirely based on their MS, they were all identified as being MDPBP, revealing the presence of a base peak at *m/z* 112 and no molecular ion peak. However, 2 of the samples shown the same R_t, different from the third one, indicating that the identified substances should be different.

A thorough analysis of the MS of the samples against the MS present iin the in-house library of synthetic cathinones confirmed the presence of MDPBP in only one of them. The other had slight differences in their MS (Figure 36).

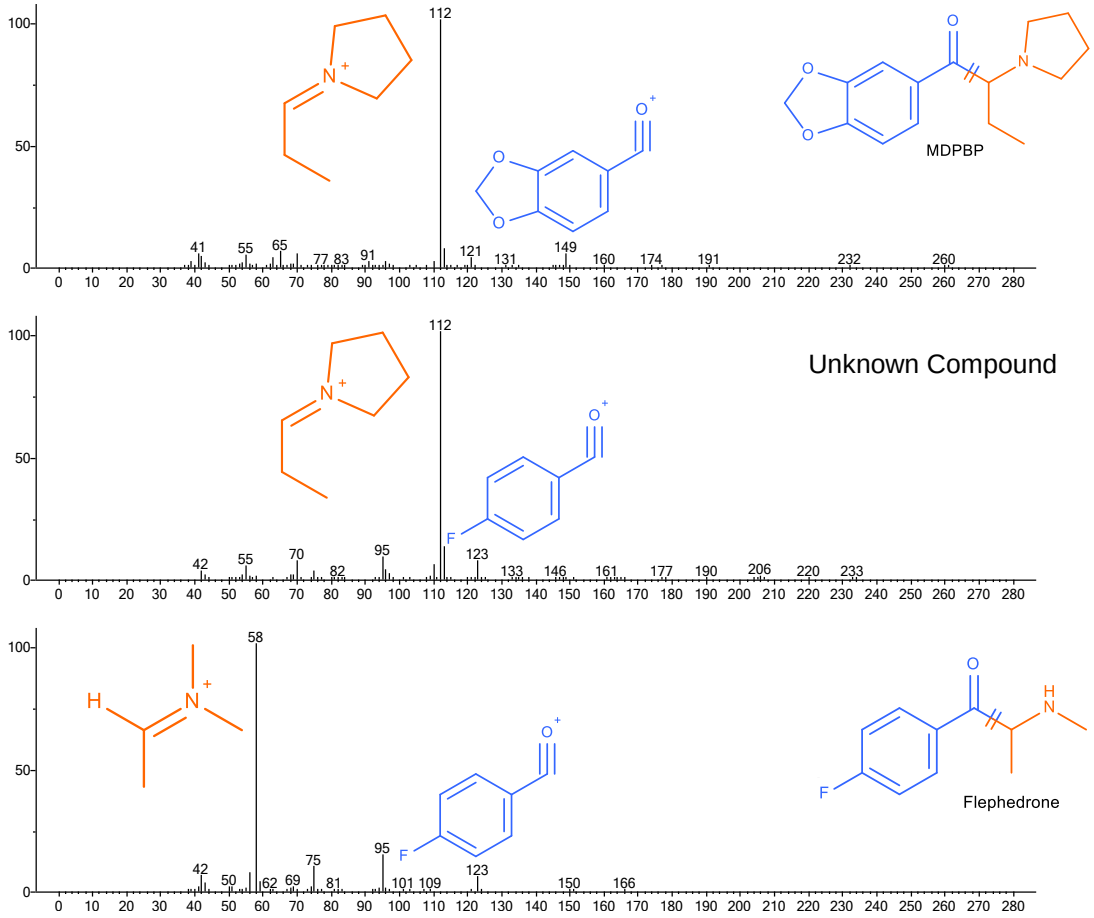


Figure 36 - Comparison of MS from Unknown Compound to MDPBP and Flephedrone

The base peak present in all three samples [*m/z* 112 ($C_7H_{14}N^+$)] is characteristic of α-pyrrolidinophenones, corresponding to the immonium ion produced by the α-cleavage of the benzyl group [58]. However, the two samples

with different R_t did not show the presence of the *m/z* 149 peak ($C_8H_5O_3^+$), characteristic of the methylenedioxybenzoyl moiety, as seen in the MS of the MDPBP standard from the in-house library. On the contrary, they shown a peak at *m/z* 123 ($C_7H_4OF^+$), which is characteristic of a fluor substitution on the benzene ring, as seen in flephedrone (see Figure 19) [41].

Taking this into account, suggestion was that the substance present in these two samples was a fluoro-substituted α-pyrrolidinobutyrophenone (Figure 37).

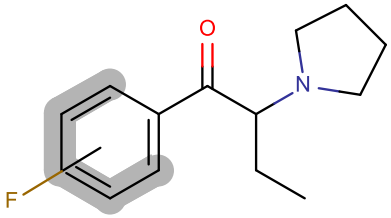


Figure 37 - Chemical structure of a fluor-substituted pyrrolidinobutyrophenone

The proposed chemical structure was elucidated and confirmed by 1D and 2D NMR tecnhiques at FCUL, within the scope of the undergraduate project of Sara Círiaco, as being 4'-fluoro-α-pyrrolidinobutyrophenone (4F-PBP) (Figure 38). The EI-MS analysis did not allow the attribution of the position of the fluor substituition on the phenyl ring. However, NMR analysis allowed the determination of the position of the fluor atom and revealed the presence of another compound, not detected by GC-MS, *myo*-inositol. (Figure 39). The structure of the coumpound was also confirmed by FTICR ($C_{14}H_{18}FNO$) at FCUL.

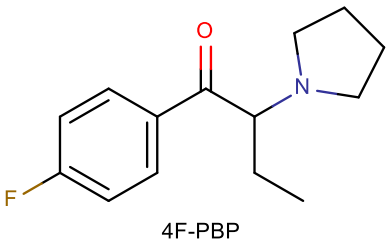


Figure 38 - Chemical Structure of 4F-PBP

The purified sample was again characterized by NMR and analysed by GC-MS, in order to be included in the in-house MS library ,as shown in Table 4

and Figure 40. As stated before, these compounds do not need to be purified to be included in the EI-MS library.

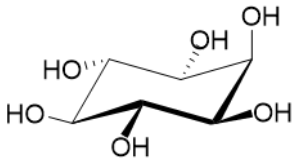


Figure 39 - Myo-inositol found in seized white powder samples

Table 4 - Addition of 4F-PBP to the in-house MS Library of Synthetic Compounds

Compound	R_t (min)	Base Peak	Other Relevant Fragmentations
4F-PBP	11,25	112	233, 123, 95

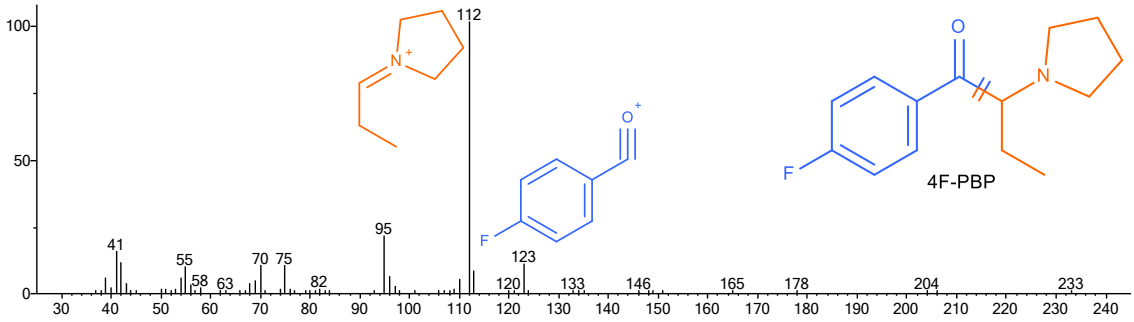


Figure 40 - EI-MS Spectrum of 4F-PBP

3.1.3. Analysis of Plant Feeders from the voluntary deliveries

The construction of the MS library did not only allow the detection of new substances, as it also permited its use in order to assess the variability of the composition of plant feeders that were catalogued from the voluntary deliveries. From the 6374 powdered plant feeders from 8 different stores, there were 16 different brand names and 17 different lot numbers. Choosing at least two samples for each lot number of each brand name, a total of 103 plant feeders were selected to analise: 73 products, with 7 different brand names (Blast, Bliss, Bloom, Blow, Charlie, Kick, Rush) equal to those used to create the MS library, and 30 space invaders with 8 diferrent brand names (Crabby, Cyclop, Darko, Demon, E.T., Mush, The Cannon, Vamp)) were analysed by GC-EI-MS [same experimental conditions as that used with the secondary standards (see section 2.5.2, page 13)] . The results were compared against the created MS in house library, in order to assess the variability within the same lot, in products from different stores, but sold under the same name and lot number (Table 5,6 and 7). The identification of the compounds present in such products was accomplished by comparison of the R_t with the in-house library and by characteristic fragmentation patterns found in MS analyses. From the 11 synthetic cathinones present in the in-house library (3,4-DMMC, 4F-PBP, 4-MEC, Buphedrone, Flephedrone, Methedrone, Methylone, MDPBP, MDPV, *N*-ethylcathinone and Pentedrone), 8 were identified in the 73 plant feeders analysed,. The compounds MDPBP, buphedrone and 4F-PBP were not detected and also no unknown compounds were seen in these analyses. In Table 5, one can see that there are qualitative variations in the composition of the compounds present in plant feeders between the same brand name or between different brands, but products do not differ qualitatively within the same lot number. However, in the case of mixtures, their relative abundances may slightly shift. This comparison of composition was based on the relative peak areas of the peaks shown in the GC-MS chromatograms. This study has allowed for the characterisation of these type of products in Portuguese smart shops. An interesting thought is that consumers were being misled when buying products under the same name, as composition could vary within the same product. Similar studies have been conducted in Portugal. Products under the

same names were analysed, with the drawback that the number of products was limited in these other studies. In 2014, Zancajo et al published their results on the analytical profile of plant feeders from smart shops in Lisbon [18]. In the same year, a research was published with plant feeders bought in portuguese smart shops from Lisbon and Porto, prior to the closing of the stores by Araújo et al [17]. In most cases, the compounds detected do not vary between the three studies, like “Blast”, “Bloom” and “Rush” products. In the case of Zancajo et al, it is difficult to succeed in a comparison, as there are no lot numbers described and the number of samples is very limited. Nevertheless, probably the most peculiar cases are products “Charlie” and “Kick”. In the two published studies, the presence of buphedrone and *N*-ethylcathinone is shown. However, all “Charlie” samples analysed in this study show no buphedrone and instead pentedrone is present in all samples. “Charlie” samples analysed by Araújo et al were from 2012, according to their lot numbers [17]. In the case of “Kick”, buphedrone mixed with caffeine was reported by Araújo et al [17]. In Table 7, it can be seen that “Kick” products from 2013 only contained pentedrone. Suggestion is that buphedrone could have been replaced by pentedrone in “Kick” and “Charlie” products, as samples from the voluntary deliveries were from 2013, according to their lot numbers. This suggestion is enforced with the literature around these two compounds. Maheux and Copeland published the chemical analysis of these two compounds in 2012, referring that pentedrone was barely detected, while buphedrone had been found in several occasions [47]; also, Giné et al refer substances detected by them up to 2012 and pentedrone is not mentioned [59]. Also, pentedrone has been mentioned on as a substitute for different synthetic cathinones. In a recent study, samples under the same name that used to contain methylone were now analysed by the DEA (Drugs Enforcement Agency), revealing the presence of pentedrone [60]. Their structural resembleance could be a motif for the replacement. Zancajo et al do not report the presence of MDPV, mixed with 4-MEC, in “Blow” products. However, “Blow” samples with no lot number analysed by Araújo et al showed the same composition as the ones presented within [17], [18]. The analysis of these products and comparison with the existing literature could lead to a profile of these products in Portugal.

Table 5 – Relative Abundances on Plant Feeders: Comparison of lot numbers of Blast and Bliss products

Product	Lots	Smartshop	Compounds			
			Methylone	Methedrone	Flephedrone	Caffeine
Blast	2012 35 12 P	A			75%	25%
		F			59%	41%
	2012 46X12P	A			75%	25%
		F			68%	32%
	2013 06X12P	A			100%	
		F			100%	
		F			100%	
	2013 13X0912P	A			100%	
		A			100%	
	Unknown	A			72%	28%
		F			71%	29%
		F			67%	33%
Bliss	2012 06X19P	A		100%		
		H		100%		
	2012 42X16P	A		100%		
		F		100%		
	2012 87014P	G		100%		
		H		100%		
	2013 09X16P	F		100%		
		H		100%		
	2013 09X19P	F		100%		
		H		100%		
	2013 15X16P	A	100%			
		F	100%			
		H	100%			
	Unknown	A	93%			7%
		C	95%			5%
		G	93%			7%

Table 6 – Relative Abundances on Plant Feeders: Comparison of lot numbers of Blast and Bliss products

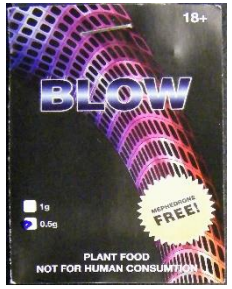
Product	Lots	Smartshop	Compounds					
			4-MEC	MDPV	Pentedrone	N-ethylcathinone	Methedrone	Methylone
Bloom 	2013 2X19P	F			54%	26%	20%	
		G			51%	35%	14%	
	2013 5X19P	G			54%	26%	20%	
		G			50%	28%	22%	
	2013 09X19P	F			47%	35%	18%	
		F			47%	30%	23%	
		F			48%	28%	23%	
	2013 15X19P	A			47%	27%		26%
		F			45%	26%		29%
		F			47%	32%		21%
	2013 16X19P	A			42%	5%		53%
		F			42%	5%		53%
Blow 	Unknown	A	83%	17%				
		A	87%	13%				
		C	96%	4%				
		C	87%	13%				
		F	90%	10%				
		F	88%	12%				
		F	94%	6%				
		F	89%	11%				
Charle 	2013 14X17P	A			51%	49%		
		C			55%	45%		
		F			55%	45%		
	2013 15X17P	A			52%	48%		
		C			53%	47%		
		F			53%	47%		
		F			52%	48%		
		F			53%	47%		

Table 7 - Relative Abundances on Plant Feeders: Comparison of lot numbers of Kick and Rush products

Product	Lots	Smartshop	Compounds		
			Pentedrone	N-ethylcathinone	Caffeine
Kick 	2013 14XT5P	A	100%		
		F	100%		
		F	100%		
		G	100%		
Rush 	2012 16X18P	A	100%		
		F	100%		
		F	100%		
	2012 42X18P	A	93%		7%
		H	92%		8%
		H	90%		10%
	2012 35018P	F	91%		9%
		F	90%		10%
		H	91%		9%
		H	92%		8%
	Unknown	A	86%	7%	6%
		F	88%		12%
		F	89%		11%

Apart from the plant feeders already discussed, which consisted on products with the same brand name than those used for the construction of the in-house EI-MS library of synthetic cathinones, there were other products labelled as powdered plant feeders that were rather curious. One of the products initially used to build the MS library was called “Crabby” and was also advertised as “Space Invader”.From the voluntary deliveries, other products were part of what seemed a special edition under the name “Space Invader” (Figure 41).

Figure 41 - "Space Invaders" Collection

This collection consisted of 10 different packages, labelled from 1/10 to 10/10. As in the case of the other plant feeders presented before, the variablity within “Crabby” products was assessed. However, as there were other products under this “collection”, it was decided to also evaluate their variability and possibly check for new substances that could be present in such products.

Table 8 - Products from the "Space Invaders" collection

Collection’s Number	Product Name
1	E.T.
2	Vamp
3	Crabby
4	Demon
6	Darko
7	Mush
8	The Cannon
10	Cyclop

Although the names of the products change, the lot numbers on the packages were independent of the name. Actually, the packages seemed to be pre-packed and afterwards sticked with a label differentiating between them. Hence, the results of the analysis were dependent on lot number and the packaging had little to do with the variance shown. Therefore, results are shown for the different lot numbers, without taking into consideration the product names (Table 9).

Table 9 - Relative Abundances on "Space Invaders" Plant Feeders: Comparison of lot numbers

Lots	Product	Smartshop	Compounds		
			3,4-DMMC	Methylphenidate	N-ethylcathinone
2012 45X28P	E.T.	F			100%
	E.T.	H			100%
2012 46X13P	E.T.	F		100%	
	E.T.	F		100%	
	Darko	F		100%	
	Darko	F		100%	
	Demon	F		100%	
	Demon	F		100%	
	Mush	F		100%	
	Mush	F		100%	
	Cannon	F		100%	
	Cannon	F		100%	
	E.T.	F		100%	
	Darko	F		100%	
	Mush	F		100%	
	E.T.	F		100%	
2012 46X28P	E.T.	F		100%	
	E.T.	F		100%	
2012 47X28P	Crabby	C	100%		
	E.T.	C	100%		
	Vamp	C	100%		
	E.T.	F	100%		
	Vamp	F	100%		
	E.T.	F	100%		
	Vamp	G	100%		
	Crabby	H	100%		
	E.T.	H	100%		
2013 06X28P	Cyclop	C	100%		
	Cyclop	F	100%		
	Cyclop	G	100%		

30 “Space Invaders” products, consisting of at least two products of each lot of each package name, were analysed. Like the other products, they were compared against the in-house EI-MS library. From this comparison, it was possible to identify 3,4-DMMC and *N*-ethylcathinone in some of the samples. However, in most of the samples (16 samples), there was no positive match with any of the compound from the library. Hence, the MS of these products were compared against the SWGDRUGS library, using the PBM search method. In this search method, reference spectra and unknown spectrum are usually compared by forward search, meaning that the fragments of the unknown spectrum must be present in the reference spectra [34]. It is therefore essential to eliminate any background signals that may exist. The difference on a MS after subtraction of the background can make a great difference in identification.

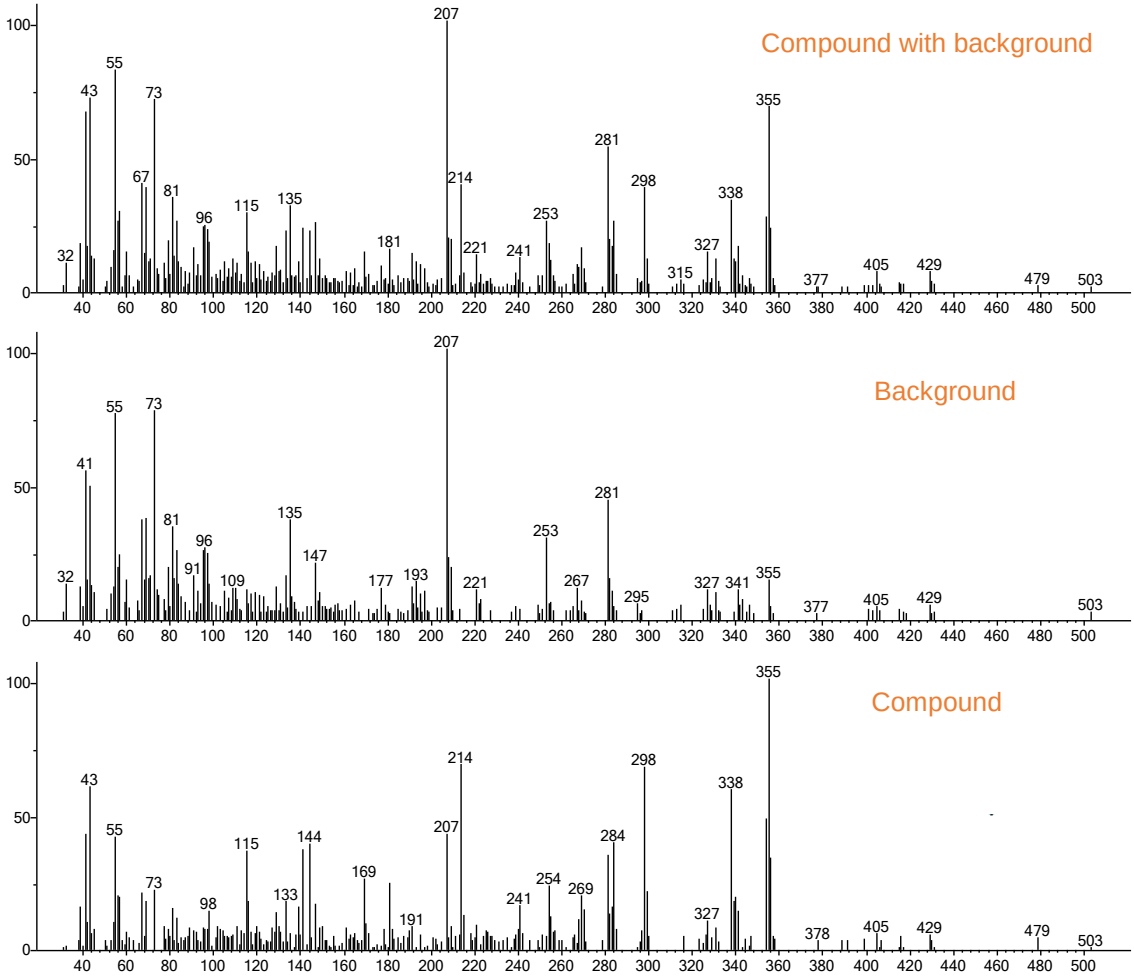


Figure 42 - Effect of the subtraction of the background from a MS

As seen in Figure 42, the subtraction of the background allowed the correct assignment of the base peak and changes in relative abundances of other peaks. One of the biggest disadvantages of the PBM search method is the fact that it is very much dependent on the data acquisition parameters of the instrument and the choice of the region of the peak. This leads to different spectra per substance in this search method [34]. Regardless, this method has allowed for the identification of a substance that showed the same R_t in all the samples (around 12.5min). In Figure 43, the first two compounds with higher matches with the library used are shown. Based on the quality percentages assigned by the search, the compound could be identified as methylphenidate.

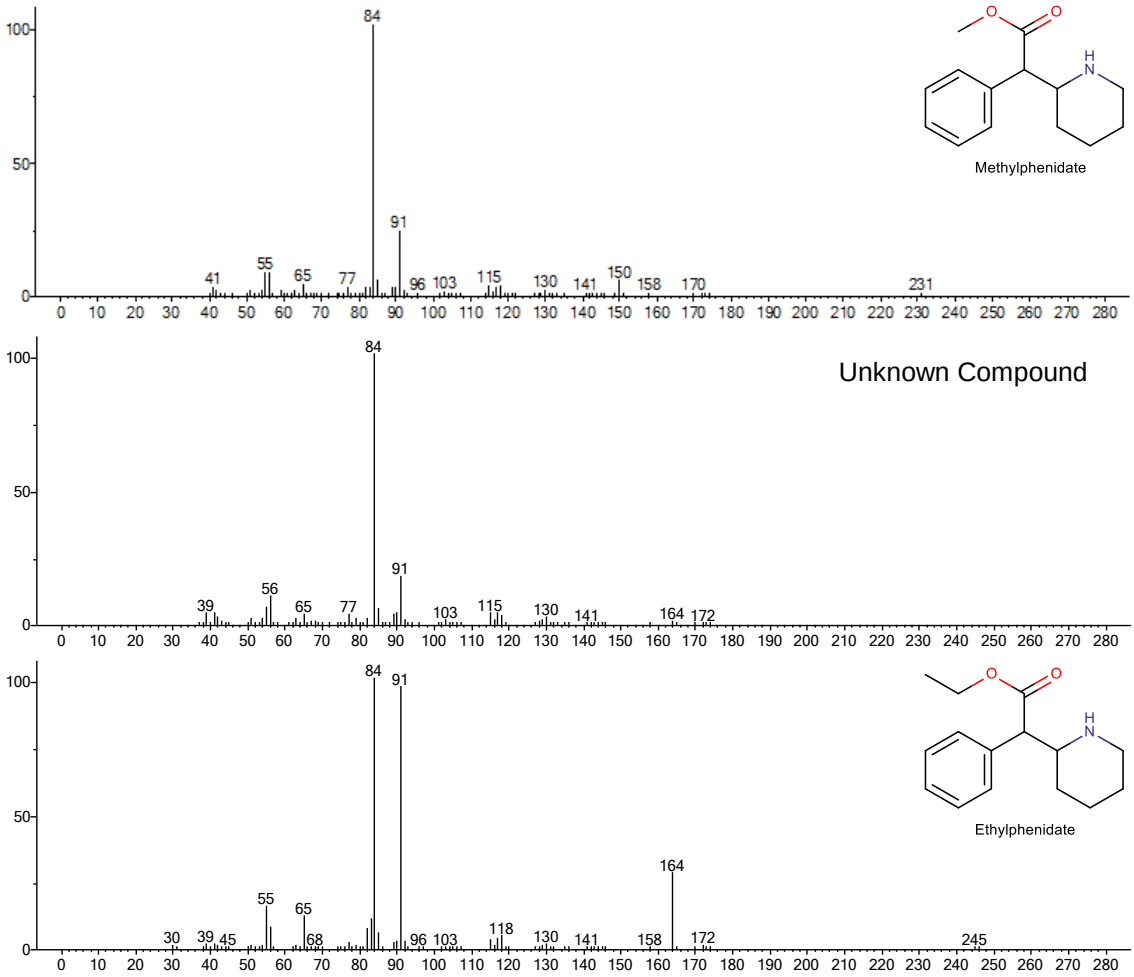


Figure 43 - PBM Search Result of sample CL93-4: Comparison with Methylphenidate and Ethylphenidate standards from SWGDRUGS library

Although there is a differentiation on the quality percentage attributed to methylphenidate and ethylphenidate, they are very similar and a differentiation based on MS puts up a great challenge. A similar spectrometric behaviour would be expected for these compounds, as their only structural difference is the substitution on one of the sides of the carbonyl group (methyl substitution for methylphenidate and ethyl substitution for ethylphenidate) (Figure 44).

Methylphenidate is considered a stimulant medication, usually prescribed to both children and adult that suffer from attention-deficit hyperactivity disorder (ADHD) [61]. In Portugal, methylphenidate is a controlled substance under *Dec-Lei* 15/93, of 22nd of January, which criminalises the trafficking of the substances present in the table appended on the decree. However, according to its article 15, it is possible to supply methylphenidate to the general public by medical prescription [14]. However, to the best of our knowledge, there are no records of the use of methylphenidate in plant feeders. In fact, based on the principle that NPS are used to circumvent existing legislation, it would not make much sense to detect this compound in this type of products.

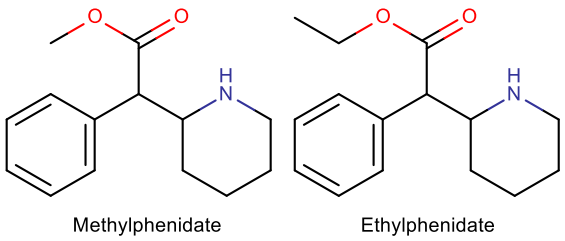


Figure 44 - Chemical Structures of Methylphenidate and Ethylphenidate

Ethylphenidate is usually found as a metabolite, after the combined ingestion of methylphenidate and ethanol, as a product of transesterification (Figure 45) [62].

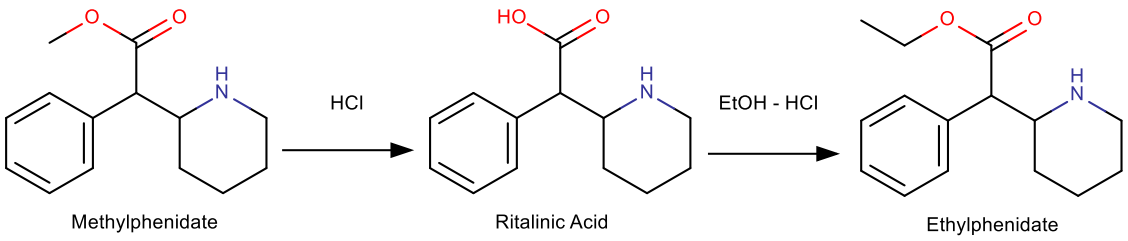


Figure 45 - Synthetic Route for Ethylphenidate (Casale and Hays, page 58) [63]

However, ethylphenidate has been found in different products sold as 'legal highs'. Casale and Hays refer the legality of the substance, although stating that, in the US, it could be considered an analogue of methylphenidate, a controlled substance. In Portugal, methylphenidate is placed on an annexed table of *Decreto.Lei 15/93* [14] that includes derivatives or analogues. Hence, ethylphenidate can be scheduled in the same legislation; despite that, this compound was put under control in *Portaria 154/2013,* under *Decreto-Lei 54/2013* [13]. As their EI-MS differentiation is complicated, the compound was analysed by NMR in order to obtain a structural elucidation of the compound.

A sample of E.T. Space Invader was analysed by NMR in D_2O. Analysis of the ^1H NMR and HSQC spectra of the unknown compound in D_2O revealed the presence of 20 protons: one methyl signal at δ1.14 (3H, t, *J*=7.2Hz); two methine signals at δ3.79 (1H, td, *J*=10.0/2.4Hz) and δ3.95 (1H, d, *J*=9.2Hz); five methylene signal at δ1.37/δ1.59 (2H, m/m), δ1.40/1.79 (2H, m/m), δ1.59/δ.1.84 (2H, m/d, *J*=14.4Hz), δ3.04/δ3.42 (2H, td/brd, *J*= 2.8/12.8Hz / *J*=12.8Hz) and δ4.18 (2H, m) and five aromatic protons at δ7.29 (2H, d, *J*=6.8Hz) and δ7.42 (3H, m). ^{13}C APT NMR spectrum revealed the presence of 15 carbons, suggesting a carbonyl carbon at δ172.71; one aromatic quaternary carbon at δ133.29; five aromatic methine carbons at δ128.58 (2 carbons), δ128.79 and δ129.42 (2 carbons); two methine carbons at δ53.79 and δ57.83; five methylene carbons at δ21.24, δ21.73, δ26.16, δ45.54 and δ62.97; and one methyl carbon at δ12.98.

1D and 2D correlation techniques allowed for the correct assignment of the signal, as seen in Figure 46 and Table 10.

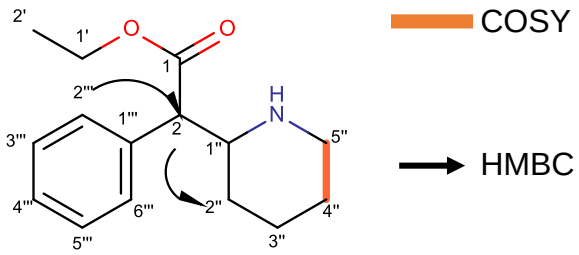


Figure 46 - Key COSY and HMBC correlations in Ethylphenidate

Attribution of the signals for ethylphenidate started with the quaternary carbon at δ_C172.71 (position 1), characteristic of a carbonyl carbon. The

methine group at C2 (δ_H3.95, d, *J*=9.2Hz; δ_C57.83) showed an HMBC correlation with the aromatic methine protons at C2''' and C6''' (δ_H7.29, d, *J*=6.8Hz; δ_C128.58), also allowing the attribution of C1'' (δ_H3.79, td, *J*=10.0/2.4Hz; δ_C53.79). The other aromatic positions were attributed as C3''' and C5''' (δ_H7.42, m, overlapped; δ_C129.42). The other *N*-methylene group at C5'' (δ_H3.04/δ_H3.42, td/brd, *J*= 2.8/12.8Hz/*J*=12.8Hz) showed a COSY correlation with the methylene group at C4'' (δ_H1.59/δ_H1.84, m/d, *J*=14.4Hz; δ_C21.73), allowing their attribution. Positions 2'' and 3'' were differentiated by an HMBC correlation between the methine group at C2 and the methylene protons at C2''. The methyl group (δ_H1.14, t, J=7.2Hz; δ_C12.98) and the methylene group (δ_H4.18, m; δ_C62.97) were assigned as C2' and C1', respectively.

Table 10 - ^{1}H and ^{13}C NMR signals of Ethylphenidate

Position	^{13}C NMR	^{1}H NMR
1	172.71	-
2	53.79	3.95, d, *J*=9.2Hz
1'	62.97	4.18, m
2'	12.98	1.14, t, *J*=7.2Hz
1''	57.83	3.79, td, *J*=10.0/2.4Hz
2''	26.16	1.59m, 1.37m
3''	21.24	1.79m; 1.40m
4''	21,73	1.84d, *J*=14.4Hz; 1.59m
5''	45.54	3.42 brd, *J*=12.8Hz; 3,04td, *J*= 2.8/12.8Hz
1'''	133.29	-
2'''/6'''	128.58	7.29, d, *J*=6.8Hz
3'''/5'''	129.42	7.42, m, overlapped
4'''	128.79	7.42, m, overlapped

The compound was elucidated as being ethylphenidate and was analysed by GC-EI-MS in order to include it in the in-house EI-MS library.

Table 11 - Addition of Ethylphenidate to the EI-MS Library of Synthetic Compounds

Compound	R_t (min)	Base Peak	Other Relevant Fragmentations
Ethylphenidate	12.56	84	146, 91, 77

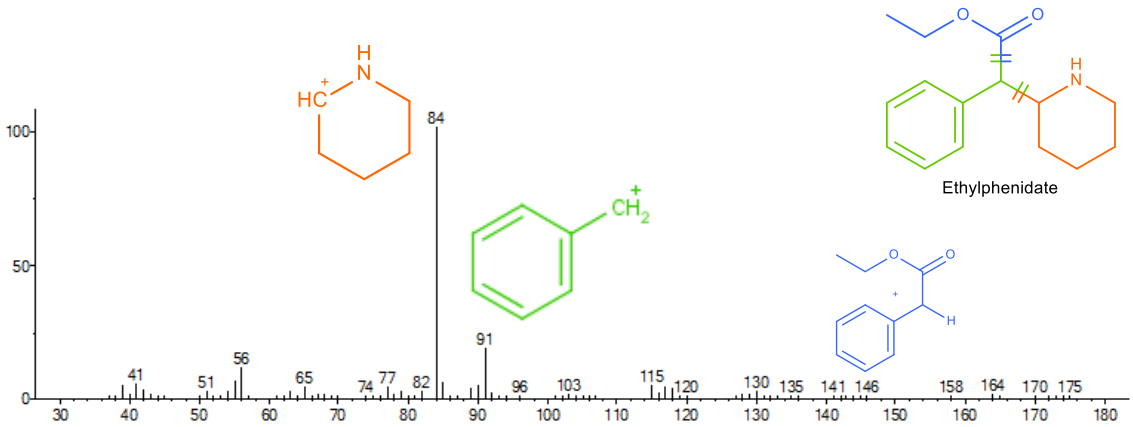


Figure 47 - EI-MS of Ethylphenidate

The EI fragmentation of ethylphenidate gives a base peak of *m/z* 84 ($C_5H_{10}N^+$), resulting from the formation of a piperidinium ion. Also a "second" base peak could be the tropylium ion at *m/z* 91 ($C_7H_7^+$), formed by cleavage with the carbonyl group, after loss of the piperidinium ion. As discussed before, the tropylium ion can undergo further fragmentation, forming the carbenium ion at *m/z* 77 ($C_6H_5^+$), present in the spectrum. The piperidinium ion can also suffer a secondary fragmentation, resultant from a transannular cleavage. This fragmentation is characteristic of saturated heterocyclic compounds, and, fundamentally, consists on a cut across the ring structure [35]. In the case of a piperidine structure, it consists on the loss of an imine (*m/z* 29), resulting in a peak with *m/z* 56. However, these fragmentations are common to both methylphenidate and ethylphenidate. Nevertheless, there is a minor fragmentation that could be used to distinguish between these two compounds. In Figure 47, a peak at *m/z* 146 ($C_{10}H_{12}O^+$) is pointed out. This peak is considered the complementary peak after the formation of the piperidinium ion, hence standing out the ethyl phenylacetate moiety. In a spectrum of methylphenidate, a peak at *m/z* 150 should be expected (Figure 47) [63].

3.1.4. Analysis of seized samples of plant feeders

Following the analyses of plant feeders from the voluntary deliveries and the assumption that products under the same name could have different compositions over time, results from samples seized from smart shops in 2011 (prior to the new legislation and the closure of the stores) with the same brand name as those previously discussed, were looked at. This was done in order to verify the existence of any other substances that could be present in the market before closure of the smart shops and, hence, add more compounds to the EI-MS library.

From this search, a result from a “Blow” product gave a positive match with existing libraries for mephedrone. This result is of particular interest, as mephedrone is one of the most known synthetic cathinones and was the first cathinone derivative to undergo the three-step approach by the EMCDDA and Europol [53], leading to Council Decision 2010/759/EU of 2 December 2010 on submitting 4-methylmethcathinone (mephedrone) to control measures, by which all Member States should submit this compound to control and criminal penalties [64]. In Portugal, mephedrone was added to *Decreto Lei 15/93* in its nineteenth amendment, with Law no 13/2012, of 26 of March. Recently, a decision was made in the UN Commission on Narcotic Drugs to place mephedrone in Schedule I of the 1971 Convention.

The structural characterisation of this compound was confirmed by NMR, using D_2O as the solvent. Analysis of the ^{1}H NMR spectrum of mephedrone in D_2O revealed the presence of 14 protons: three methyl signals at δ1.62 (3H, d, *J*=7.2Hz), δ2.45 (3H, s) and δ2.82 (3H, s); a methine signal at δ5.09 (1H, d, *J*=7.2Hz) and four aromatic protons at δ7.45 (2H, d, *J*=8.0Hz) and δ7.93 (2H, d, *J*=8.4Hz). ^{13}C APT NMR spectrum revealed the presence of 11 carbons, suggesting a carbonyl carbon at δ197.95, two aromatic quaternary carbons at δ130.50 and δ148.24; four aromatic carbons at δ129.90 (2 carbons) and δ130.74 (2 carbons); a methine carbon at δ60.37 and three methyl carbons at δ16.27, δ21.83 and δ31.76.

1D techniques and 2D correlation techniques allowed for the attribution of the signals, as seen in Figure 48 and Table 12.

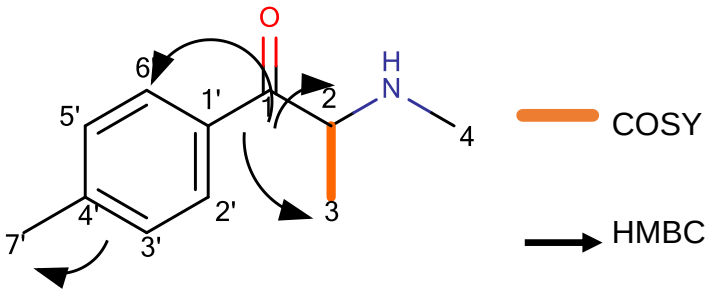


Figure 48 - Structure of Mephedrone and key COSY and HMBC correlations

Attribution of the signals for mephedrone started with the quaternary carbon at δ_C197.95 (position 1), characteristic of a carbonyl carbon. C1 showed HMBC correlations with protons at positions 2 (δ_H5.09, d, *J*=7.20Hz δ_C60.37;), 3 (δ_H1.62, d, *J*=7.20Hz; δ_C16.27) and 6'/2' (δ_H7.93, d, *J*=8.40Hz; δ_C130.74;), allowing their correct assignment. Also, H2 showed a COSY correlation with the mehyl at δ1.62 atributed to C3. The other aromatic signals (δ_C129.90; δ_H7.45, d, *J*=8.00Hz) were attributed to positions 3' and 5' by the COSY correlation beteween δ_H7.93 (H6'/H2') and δ_H7.45.

Table 12 - ^{1}H (400.13MHz) and ^{13}C (100.6MHz) spectral data of Mephedrone recorded in D_2O

Position	^{13}C NMR	^{1}H NMR
1	197.95	-
2	60.37	δ5.09 (1H, d, *J*=7.2Hz)
3	16.27	δ1.62 (3H, d, *J*=7.2Hz)
4	31.76	δ2.82 (3H, s)
1'	130.5	-
2'/6'	130.74	δ7.93 (2H, d, *J*=8.4Hz)
3'/5'	129.90	δ7.45 (2H, d, *J*=8.0Hz)
4'	148.24	-
7'	21.83	δ2.45 (3H, s)

HMBC correlation between a quaternary carbon (δ_C148.24) and a methyl group (δ_H2.45, s; δ_C21.83) permitted the attribution of 4' and 7', respectively. With an aromatic carbon and a methyl group left, attribution of 1' and 4 as δ_C130.50 and (δ_C31.76; δ_H2.82, s) was possible.

After structural elucidation by NMR, the product was analysed by GC-MS, using the same methodology used for the other plant feeders. Because the compound could be identified by NMR as being mephedrone, this analysis was

conducted in order to add a R_t and a MS for this compound to the in-house library (Figure 49 and Figure 50).

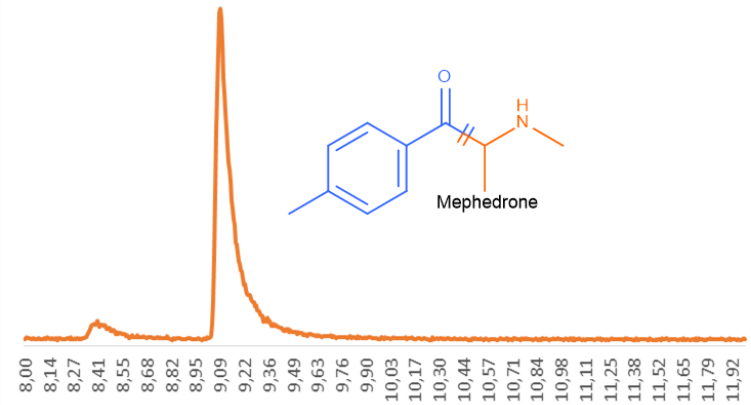


Figure 49 – Retention Time of Mephedrone

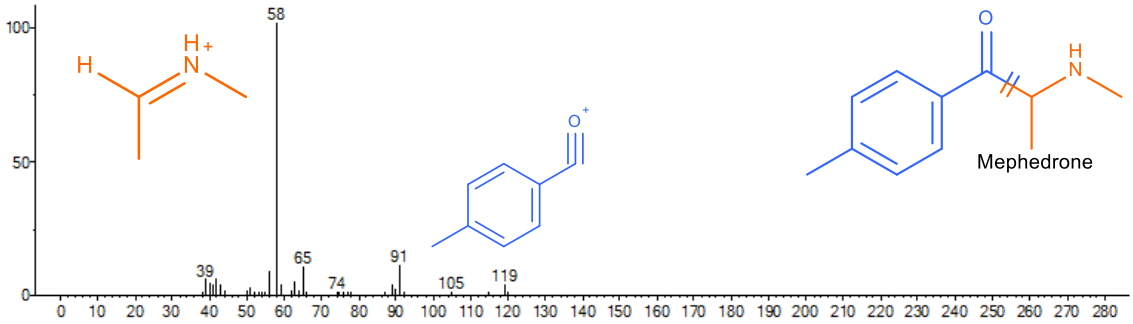


Figure 50 - EI-MS of Mephedrone

The spectrometric behaviour is the expected for a methyl substituted methcathinone, as seen in other cases discusses above. Characteristic iminium and acylium ions reflect the substitution patterns, as well as secondary fragments, which were already presented and discussed in Figure 24.

The analysis of 115 samples of plant feeders (8 initial standards, 103 products from voluntary deliveries and 4 seized sample) has allowed the construction of an in-house EI-MS library of 12 synthetic cathinones (3,4-DMMC, 4-MEC, 4F-PBP, Buphedrone, Flephedrone, MDPBP, MDPV, Mephedrone, Methedrone, Methylone, *N*-ethylcathinone and Pentedrone) plus ethylphenidate (Figure 51). This is of the uttermost importance for a routine laboratory, as the thorough study of the spectrometric and fragmentation behaviour of these compounds may aid in the discovery of novel NPS and in the continuous monitoring of already existing substances. Common fragmentations make them easier molecules to assess and the advantage of an R_t facilitates the correct identification on these compounds. Indeed, the methodology used and the in-house library allowed the identification of a new synthetic cathinone in Europe, 4F-PBP, firstly suggested by MS comparison with the secondary standards and then confirmed by NMR analyses. The variability studies permitted the study of the qualitative and quantitative variations of synthetic cathinone in Portugal. An interesting remark is that, of the more of 70 synthetic cathinones already reported in the EU [65], only 12 could be found in Portuguese smart shop from 2011 to 2013, plus ethylphenidate and caffeine.

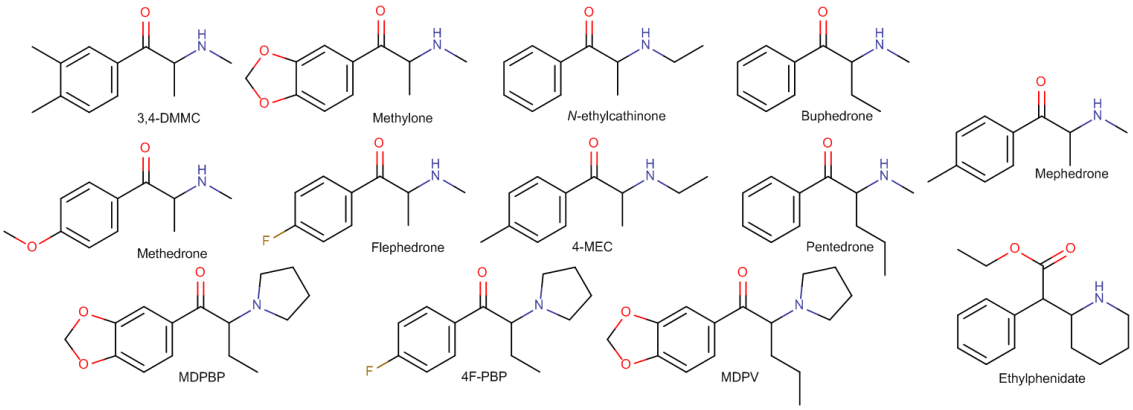


Figure 51 - Final In-house EI-MS Library with Compounds from Plant Feeders

3.2. Mass Spectrometry Library of Synthetic Cannabinoids

3.2.1. Preliminary GC-EI-MS analysis

In order to build an in-house library that contained more than synthetic cathinones, herbal incenses, known to contain synthetic cannabinoids, were analysed. Of the 15177 herbal incenses products, there were only 33 different brand names (2012, Algerian Blend, Apple, Atomic Bomb, Blow, Bombastic Kaboom Spliff Atomic Bomb, B.R.O.S., Buddah, Butterfly, Caramba, Cheese, Esfinge, Freemind, Future, Home Run, Kaboom, Magic, Mandala, MÁUI, Maya2012, Planet H, PUM!, Radioactive, Rainbow, Red Sunshine, Royal Mix, Smoke, So High, Spike99, Spliff, T-Rex, The Unicorn, Tornado, Whacked). One package of each brand name was analysed by GC-MS, in order to do a preliminary identification of the compounds present in the products.

As seen in the Introduction section (section 1.4.), these products are known to contain synthetic cannabinoid receptor agonists. Hence the GC-MS method chosen, a recommended method by the UNODC, which is destined to forensic expert laboratories, in order to qualitatively analyse such substances [28]. In Table 14, the identification of the compounds present in 23 of the 33 products is shown [in 10 products (Algerian Blend, Apple, Bombastic Kaboom Spliff Atomic Bomb, B.R.O.S., Cheese, Home Run, Planet H, PUM!, Red Sunshine, Smoke and Tornado) no compound was detected]. The identification of these compounds was based in a PBM search against known EI MS spectra libraries, like the SWGDRUG or ENFSI DRUGS 2015 [34]. This initial analysis revealed the presence of 10 different synthetic cannabinoids, as well as other compounds, such as MDPV (a synthetic cathinone already found in herbal incenses [66]), vitamin E (a natural metabolite usually found in the plant used as matrix in herbal incense products [10]) and caffeine (a common adulterant of NPS) and 5-MeO-DALT (Figure 52), which is a psychoactive substance, derivative of the natural occurring monoamine alkaloid, tryptamine. This substance was first synthesised by Alexander Shulgin [67]. Up to 2012, there had been some reports of the use of this substance in herbal mixtures, although it was more commonly found in capsules or as a white powder [68].

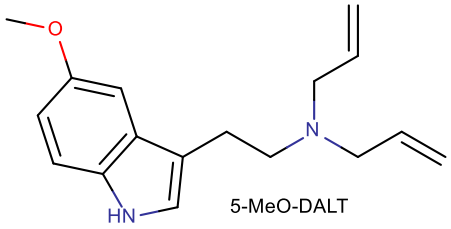


Figure 52 - Chemical Structure of 5-MeO-DALT

Herbal Incense products where caffeine was present were curious. Blow, Future and Butterfly were advertised as herbal incenses. However, the contents of the packages were white powders. In any case, samples were prepared following the same methodology applied to herbal incenses, in order to investigate the presence of any synthetic cannabinoid in these products. Initial analysis by GC-EI-MS revealed the presence of caffeine. The obtained MS is shown as an example of the results.

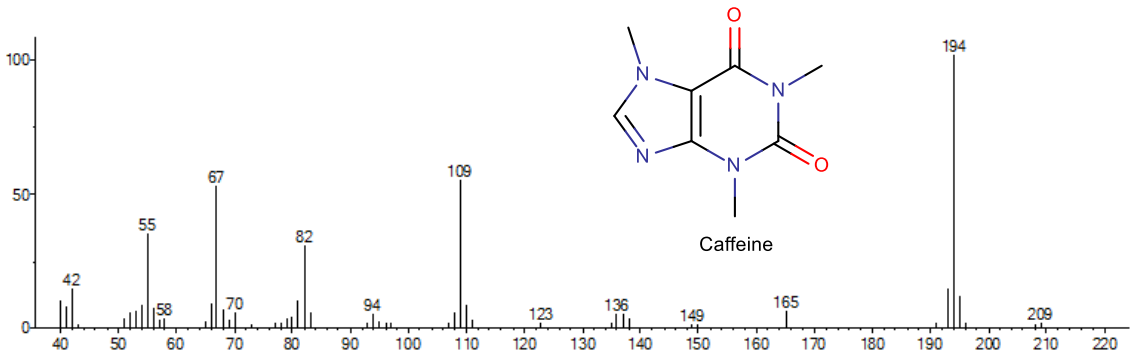


Figure 53 - EI-MS of Caffeine

The primary analysis of the synthetic cannabinoids detected is shown in Table 13.

Table 13 - GC-EI-MS Results of Preliminary Analysis

Compound	Number of Detections	R_t (min)]	Base Peak	Other EI Fragmentations	Library; PBM Search Match %
JWH-210	9	[16.4-16.6]	369 [M^+]	312, 298, 214, 183, 144	SWGDRUG; [86-99]
JWH-018	6	[14.4-14.5]	341 [M^+]	284, 270, 214, 155, 144, 127	SWGDRUG; [93-99]
JWH-122	12	[15.8-16.0]	355 [M^+]	298, 284, 214, 169, 144, 141	SWGDRUG; [89-99]
JWH-250	6	[11.4-11.7]	214	335 [M^+], 214,144, 121, 91	SWGDRUG [83-99]
MAM2201	1	17.2	373 [M^+]	298, 284, 232, 144, 141, 115	SWGDRUG, 99
AKB-48	2	[13.7-13.9]	215	365 [M^+], 308, 294, 187, 135	SWGDRUG, [97-99]
AM-694	2	13.6	232	435[M^+], 360, 220, 204, 144	SWGDRUG, 97
Cl-AM-694	2	16.0	248	451 [M^+], 360, 220, 204, 144	SWGDRUG, 95
AM-1248	2	17.9	98	390 [M^+], 292, 135, 98, 70	SWGDRUG, 83%
UR-144	1	7.0	214	311 [M^+], 144	SWGDRUG, 99

Table 14 - Preliminary GC-MS Identification of Compounds present in 23 Herbal Incenses

Product	Compound											
	JWH-210	JWH-018	JWH-122	MAM2201	JWH-250	AKB48	UR-144	AM-694 chloro derivative	AM-694	AM-1248	Caffeine	Other
Esfinge	X											
MÁUI	X	X	X	X								[1]
Caramba	X		X									[1]
Mandala		X	X									
Buddah	X	X	X									[1]
2012	X	X	X									[1]
Atomic Bomb												[2]
Kaboom					X*							
Blow											X	
So High			X		X*							
Future											X	
Rainbow	X				X							
Butterfly											X	
T-Rex												[2]
Spliff			X		X							
The Unicorn	X		X		X							
Whacked						X						
Royal Mix	X		X		X							
Maya2012						X	X					
Magic		X										
Freemind	X	X	X									
Radioactive			X					X	X	X		[3]
Spike99			X					X	X	X		[3]

* Or one of the positional isomers of JWH-250: JWH-302 or JWH-201

[1] Vitamin E

[2] MDPV

[3] 5-MeO-DALT

The initial identification of the synthetic cannabinoids present in the 23 samples of herbal incenses was achieved by comparing the obtained MS to the reference MS of the SWGDRUGS library, using the PBM method described before, the literature and by analysing their fragmentation pattern. On the next page, the obtained MS of 3 synthetic cannabinoids of the naphtoylindole family are shown. According to the match with the library, they could be identified as being JWH-018, JWH-122 and JWH-210, respectively. As they belong to the same compound family, it should be expected that their structures and, hence, their spectrometric behaviour, were similar. In fact, they not only belong to the same family, as they only differ on the substitution on the same position on naphthoyl side of the structure. As seen in Figure 54, JWH-018 is unsubstituted, JWH-122 has a methyl substitution and JWH-210 has an ethyl substitution in the *para*-position. The rest of the structure is the same in the 3 compounds.

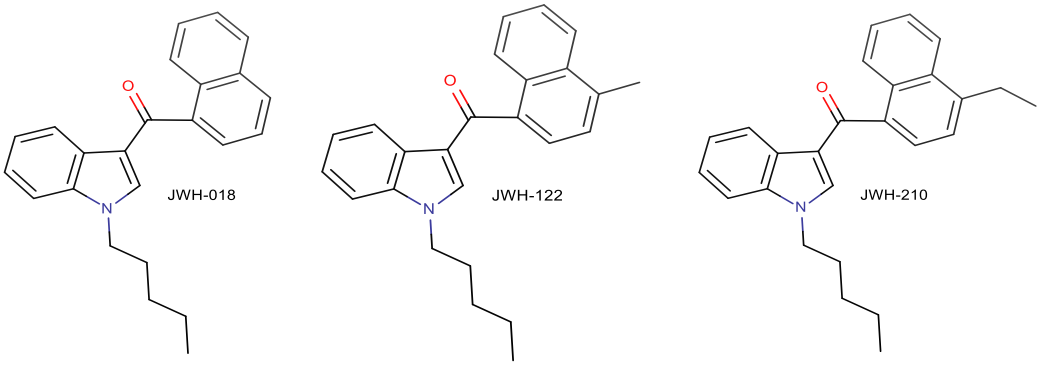


Figure 54 - Chemical Structure of JWH-018, JWH-122 and JWH-210

Unlike the case of synthetic cathinones, these structures do not undergo extensive fragmentation under EI conditions. As a matter of fact, their molecular ion is not only present in the MS, as it is, in most of the cases, the base peak [69], [70], [71]. Figure 55 shows the EI-MS spectra of the 3 compounds obtained in the GC-EI-MS analysis of herbal incenses. The main primary fragmentations of these compounds occur in the indole moiety, either with the loss of the pentyl-substitution on the nitrogen atom (Fragments B, Figure 56), the loss of the naphthalene group, with transfer of charge to the oxygen atom of the carbonyl (Fragments D, Figure 56) or with the loss of the carbonyl group, leading to a fragment with a loss of 17 Da $[M-17]^+$ [72] (Fragment A, Figure 56).

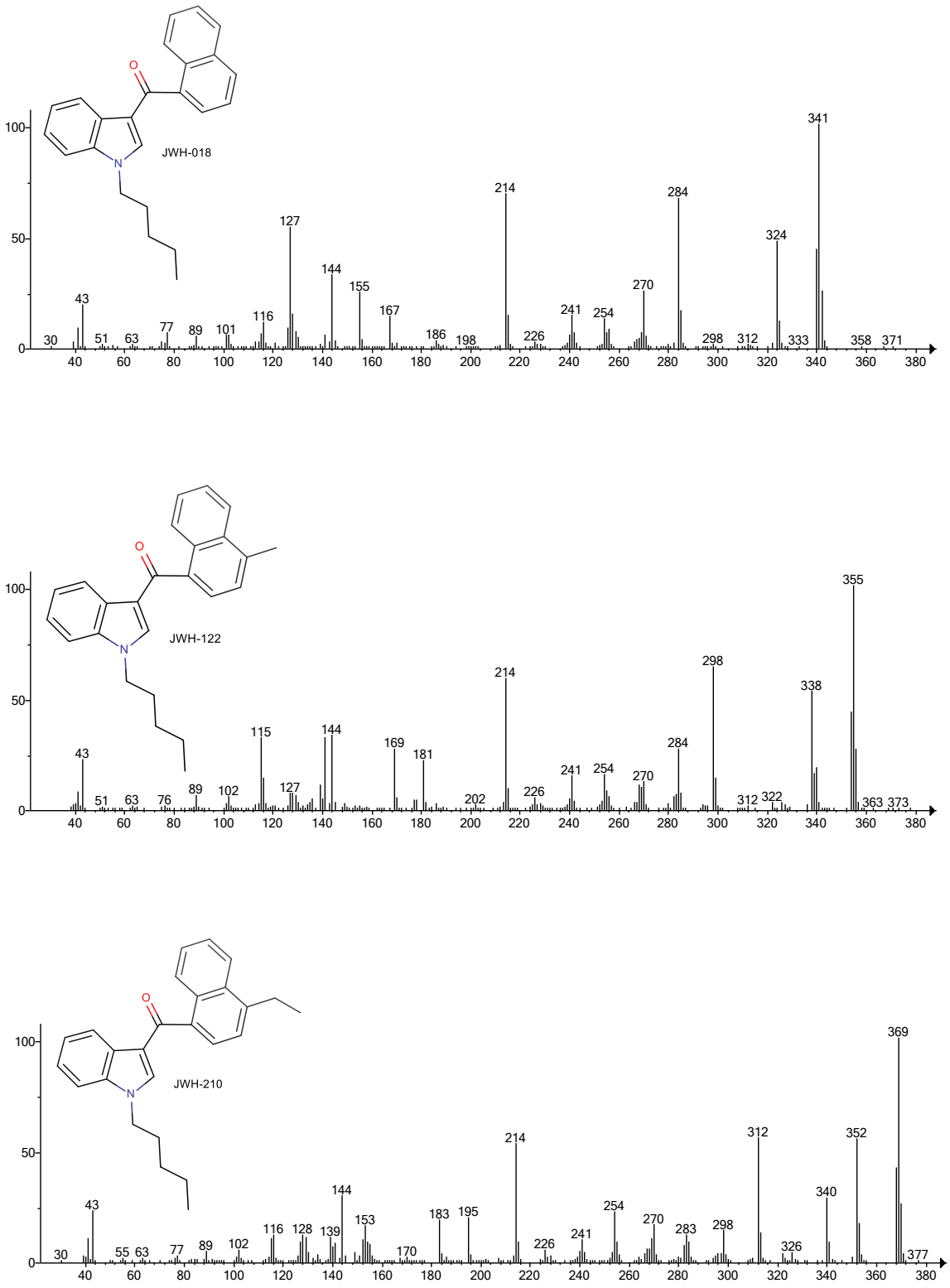
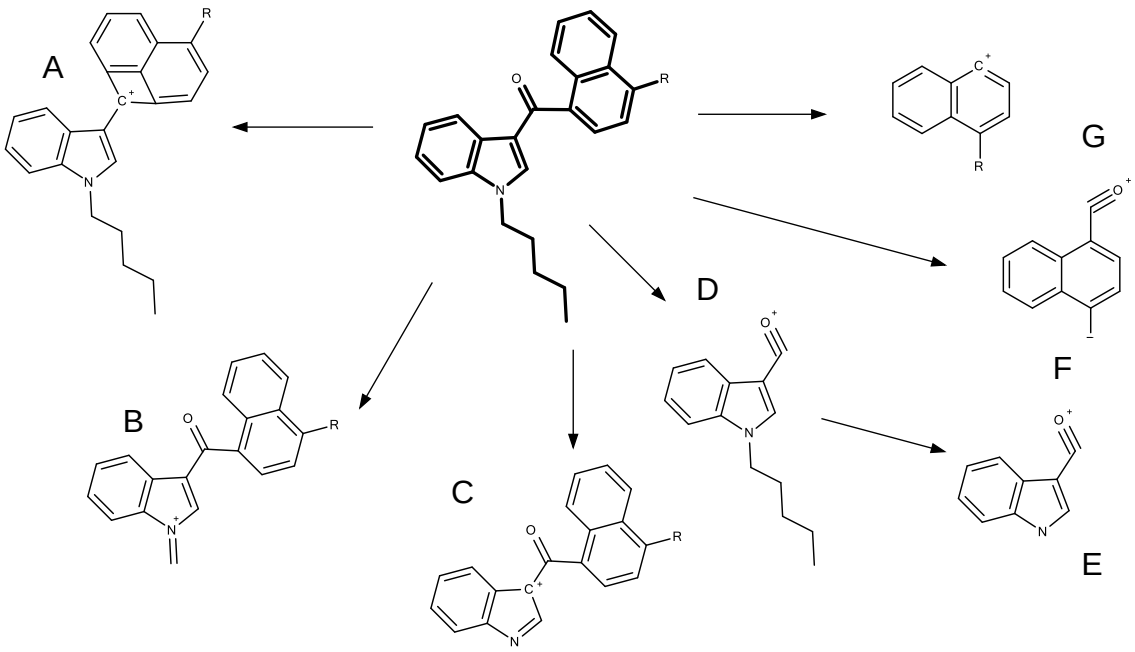


Figure 55 - EI-MS of 3 Naphthoylindoles Synthetic Cannabinoids: JWH-018, JWH-122, JWH-210



	JWH-018; R=H [73]	**JWH-122; R=CH_3** [74]	**JWH-210; R=C_2H_5** [75]
	M.W. = 341	M.W. = 355	M.W. = 369
A	324	338	352
B	284	298	312
C	270	284	298
D	214	214	214
E	144	144	144
F	155	169	183
G	127	141	155

Figure 56 - Major fragment ions in the EI-MS of 3 Naphthoylindoles: JWH-018, JWH-122 and JWH-210

Minor and secondary fragmentations occur on both sides of the molecule. Fragments F and G show characteristic naphthoyl and naphthalene fragments, respectively [76]. An interesting fragment is the secondary fragmentation E, showing the indole moiety with the charge on the oxygen of the carbonyl, without the pentyl substitution. This fragment, mainly consisting on the indole structure, could be considered a specific fragment. Loss of the carbonyl group is possible, as seen on fragment A. Therefore, it is suggested that fragment E could suffer a tertiary fragmentation, losing the carbonyl group and leading to a fragment with an *m/z* 116 ($C_8H_6N^+$), resulting from the loss of 28Da (C=12Da + O=16Da). Analysing the MS present in Figure 55, the presence of a small fragment with the *m/z* of 116 is noticeable. This is consistent with the literature, where several reports on the EI-MS behaviour of these compounds revealed the presence of a small fragment with an *m/z* 116

[70], [74], [75]. On the case of JWH-122, this smaller fragment is difficult to observe when looking at the full spectrum, due to the presence of a peak with higher intensity at *m/z* 115 [77]. As seen in the case of cathinones with a benzyl group, where the loss of an acetylene group leads to a cyclopentadienyl ion, it could be suggested that the same fragmentation occurs in compounds with a naphthyl group, as it is the case of JWH-122. Thus, the loss of an acetylene group (26Da) from the naphthyl fragment [*m/z* 141($C_{11}H_9^+$)] could lead to a fragment of *m/z* 115 ($C_9H_7^+$) [48]. The same fragments were reckoned on the analysis of the other identified synthetic cannabinoids, in order to assess the presence of other indole-type compounds or substances with a naphthoyl/naphthalene moiety.

An interesting compound is MAM2201, that could be considered the fluor derivative of JWH-122, (Figure 57) [78]. To the best of our knowledge, MAM-2201 was first reported to be isolated from a commercial product in 2012, coupled with its naphthalene unsubstituted analogue, AM-2201 [79].

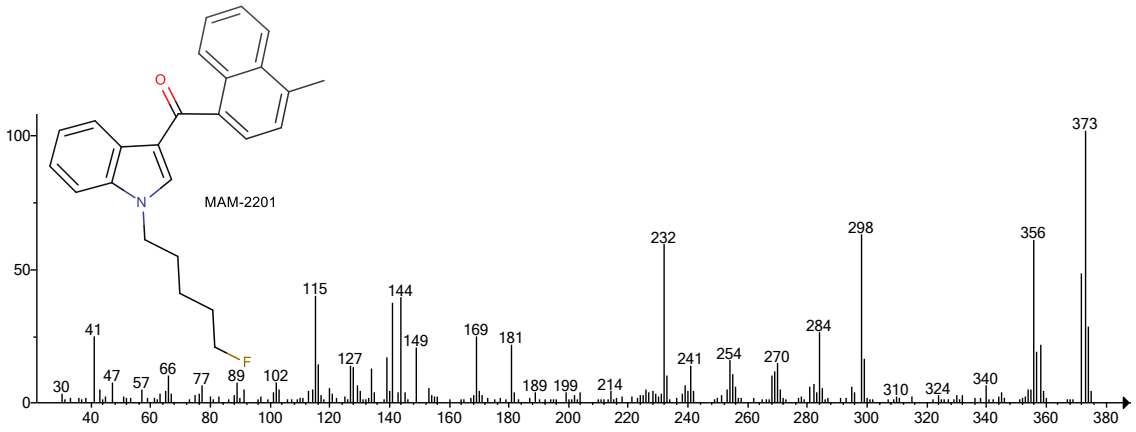
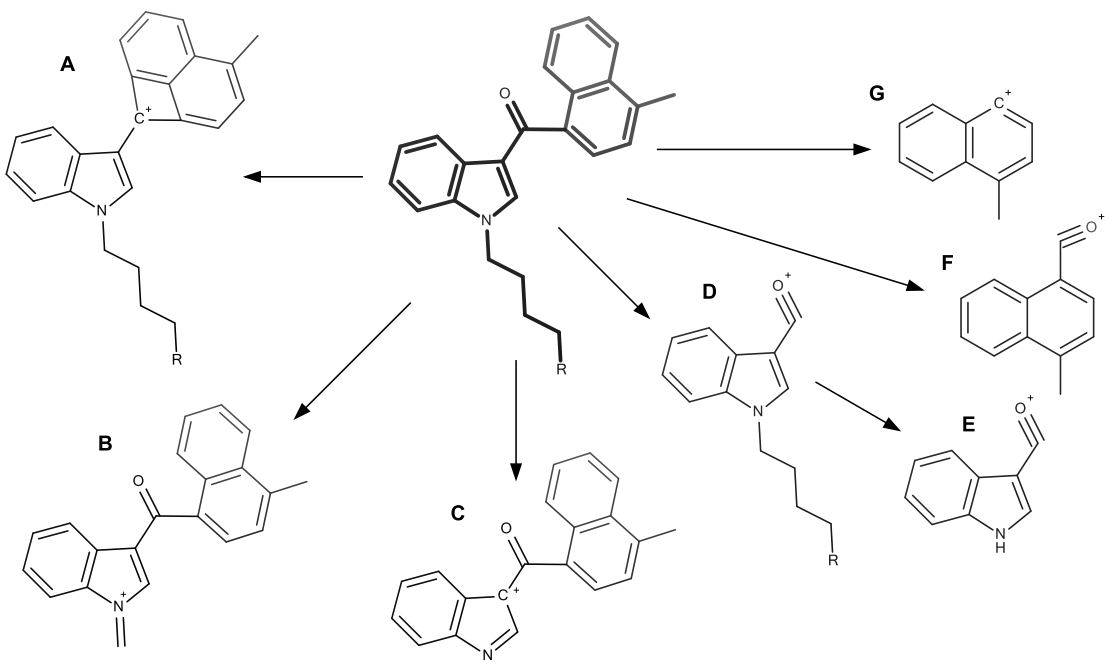


Figure 57 - EI-MS of MAM2201

As seen for the previous compounds, the base peak for MAM-2201 is its molecular ion [*m/z* 373 ($C_{25}H_{24}FNO^+$). This could be an indication of a fluor substitution on JWH-122, due to the difference of 18Da in the molecular ions [JWH-122 [M^+]: *m/z* 355 ($C_{25}H_{25}NO^+$)]. This difference should be present in all fragments were the pentyl substitution is present. Based on this premise, the spectra of MAM-2201 and JWH-122 were compared in order to assess the existence of such fragments (the same nomenclature for the fragments as seen in Figure 56).



Fragment	JWH-122 R=CH_3; M.W. =355	MAM-2201 R=CH_2F; M.W. = 373
A	338	356
B	298	298
C	284	284
D	214	232
E	144	144
F	169	169
G	141	141

Figure 58 - Comparison of Fragments of JWH-122 and MAM-2201

As seen in Figure 58, fragments A and D (where the substitution on the indole moiety is present) reveal fragments with an increase of 18Da for MAM-2201. Also, the presence of the naphthyl group can be confirmed by the presence of fragments F and G. Nevertheless, the presence of a methyl substitution on MAM-2201 can also be confirmed by the occurrence of a peak at *m/z* 115 ($C_9H_{12}^+$).

The two compounds with a pentyl-substitution on the indole moiety, but with different substituent groups on the other side of the carbonyl are JWH-250 and UR-144 (Figure 59). To our knowledge, analytical information on JWH-250 was first reported in 2011, by Nakajima et al [80], and on UR-144 by Choi et al, in 2013 [81].

Unlike the naphthoylindoles analysed before, the base peaks of JWH-250 and UR-144 are not their molecular ion. In these compounds, the characteristic fragment of a pentyl-substituted indole with the charge on the oxygen atom of the carbonyl group at *m/z* 214 ($C_{14}H_{16}NO^+$) is the base peak, with the molecular ion being almost absent of the full EI spectrum (Figure 59). Taking into consideration the fragmentation patters occurring in synthetic cathinones, where α-cleavage accounts for the main fragmentation, and considering that fragment *m/z* 214 could be assumed as an acylium ion, chances are that, in JWH-250 and UR-144, α-cleavage occurs as the main fragmentation pathway.

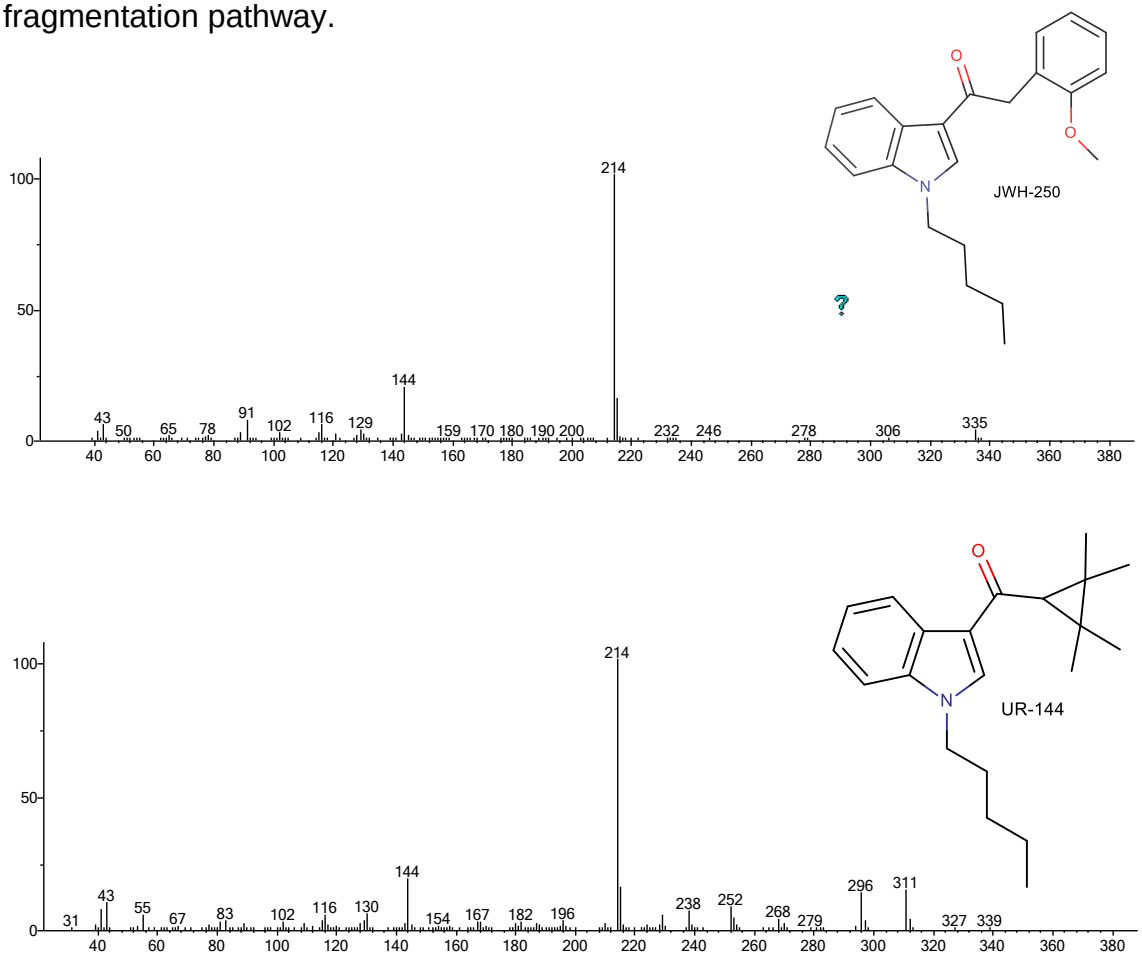


Figure 59 - EI-MS of Pentyl-substituted Indoles Synthetic Cannabinoids, JWH-250 and UR-144

The occurrence of this peak in JWH-250 was shown by Gwak et al [82]. They performed different MRM (Multiple Reaction Monitoring) analyses on this compound (and on JWH-018, JWH-122 and JWH-210) that revealed that the primary fragmentation that occurs on the JWH-250 is the formation of the peak at *m/z* 214 ($C_{14}H_{16}NO^+$). Looking at the structure of the compound, an α-carbon is present on one of the sides of the carbonyl group. Alike, in UR-144 there is

also an α-carbon. Therefore, as seen in the case of cathinone derivatives, JWH-250 and UR-144 suffer α-cleavage, leading to the formation of acylium ions. This can also be confirmed by the presence of a peak at *m/z* 144 ($C_9H_6NO^+$) in both compounds. This is formed by loss of the carbonyl group in the acylium ion, as seen in Figure 56. Although present in these compounds as their base peak, *m/z* 214 can be found in all synthetic cannabinoids with a pentyl-substituted indole moiety, as seen in Figure 60.

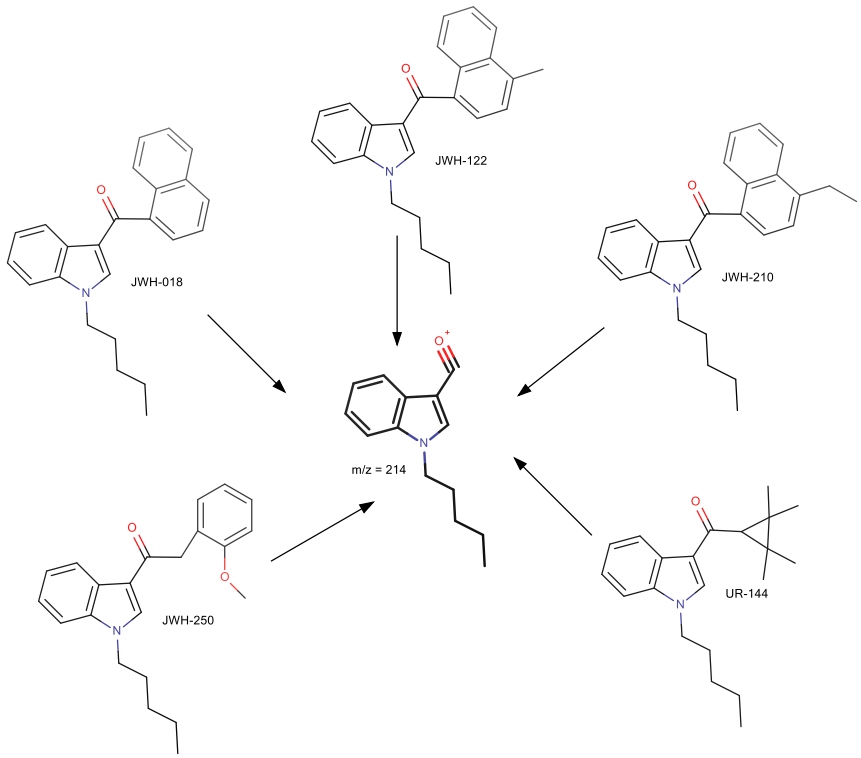


Figure 60 - Formation of peak at m/z 214 in all pentyl-substituted indole synthetic cannabinoids

JWH-250 has a characteristic moiety on the other side of the carbonyl group. Coupled to the α-carbon is an anisole group. This group is characterised by a benzene ring substituted with a methoxy group (hence, its common name methoxybenzene). Following α-cleavage, and apart from the base peak, another possible ion is the methoxybenzenium ion. This ion is shown in a peak at *m/z* 121 ($C_8H_9O^+$) (Figure 61). Although literature suggests that aromatic rings often considerably stable, this peak is barely present in the MS for this compound. However, the study by Gwak et al refers the presence of this peak. Nonetheless, the presence of an aromatic ring in this compound can be

confirmed by the peak at *m/z* 91 ($C_7H_7^+$) that, as seen before, results from benzylic cleavage of a substituted benzenium ion [34].

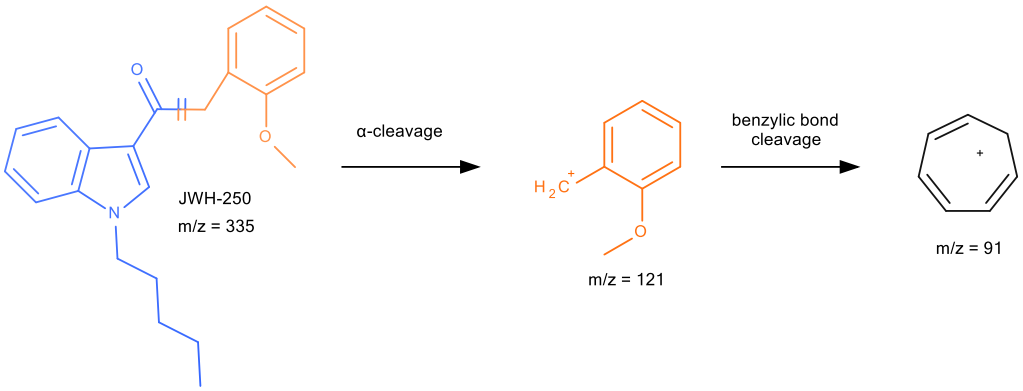


Figure 61 - EI-MS Fragmentations on the Anisole Moiety of JWH-250

Like JWH-250, UR-144 also shows an interesting moiety on the other side of the carbonyl group, a tetramethyl substituted cyclopropane ring. Cyclopropane rings are known to be thermally unstable and this poses a challenge on the EI-MS analysis of compounds with this group present. In fact, when analysing the MS shown in Figure 59, few peaks are abundant and indicative of the fragments that characterise this compound. The three major peaks are *m/z* 214 ($C_{14}H_{16}NO^+$), *m/z* 144 ($C_9H_6NO^+$) and *m/z* 311 ($C_{21}H_{29}NO^+$), the latter being the molecular ion and the first two related to the pentyl-substituted indole moiety, as described before. Hence, there are no major fragmentations characteristic of the cyclopropane ring. This is in accordance with the literature. Different reports on the GC-EI-MS analysis of UR-144 do not show any relevant peaks that could indicate the moiety on the other side of the carbonyl group [83], [84], [81]. However, taking *m/z* 311 and *m/z* 214 into consideration, suggestion is that the fragment characteristic of the other side of the carbonyl group will show a peak at *m/z* 97, resulting from the loss of 214Da from the molecular ion of 311Da. Nevertheless, there is no peak at *m/z* 97 on the MS in Figure 59. Despite that, and assuming that a tetramethyl substituted cyclopropane ring is present, a peak of this mass would be possible [*m/z* 97 ($C_7H_{14}^+$)].

Nonetheless, the fact that the cyclopropane moiety is thermally unstable yields a characteristic GC behaviour, with the appearance of a second peak, usually just immediately next to the compound of interest and that reveals a rearrangement in the cyclopropane group [83]. This peak will then be analysed by the MS as if it was an unknown compound [35]. Literature suggests that this rearrangement product is based on the opening of the cyclopropane ring [83], explained by the presence of an abundant fragment 15Da greater than the base peak of UR-144 and with the same molecular ion, as seen in Figure 61. Kennedy et al suggested that this degrading product could suffer a McLafferty rearrangement, which is possible, considering the presence of a y-hydrogen and a β-carbon [85]. The EI-MS of the UR-144 cyclopropyl rearrangement product is shown below (Figure 62).

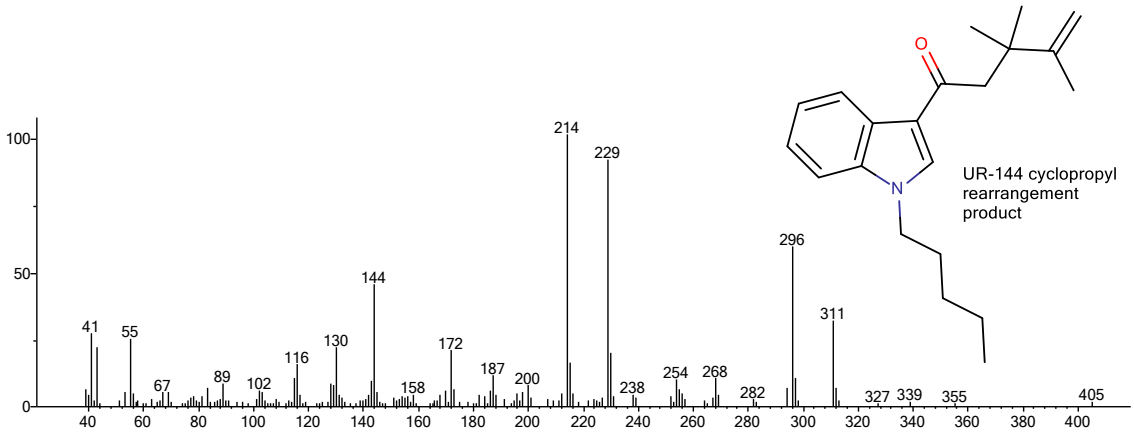


Figure 62 - EI-MS of UR-144 cyclopropyl rearrangement product

This MS shows the same molecular ion as UR-144 [*m/z* 311 ($C_{21}H_{29}NO^+$)] and the characteristic peaks of a pentyl-substituted indole moiety, *m/z* 214 ($C_{14}H_{16}NO^+$) and *m/z* 144 ($C_9H_6NO^+$) (Figure 62). The other relevant peak is the radical cation at *m/z* 229 ($C_{15}H_{19}NO^+$) that results from proton migration. This peak confirms the acyclic structure of the product, after rearrangement of the cyclopropyl moiety of UR-144 (Figure 63) [86].

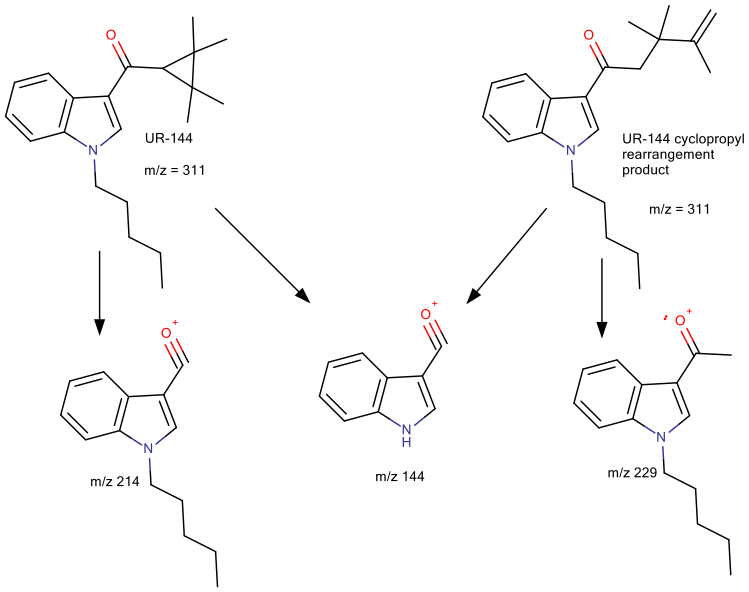


Figure 63 - Principal Fragmentations of UR-144 and its cyclopropyl rearrangement product

AM-694 and its chloro derivative were also identified in the initial analysis of herbal incenses.AM-694 was first reported in the literature as an adulterant in herbal blends by Nakajima et al in 2011 [70], but was reported to the EMCDDA via the EWS in 2010 by the Irish authorities. Its chloro derivative was first reported in 2011 by German authorities and, to the best of our knowledge, there are no reports discussing this compound in the literature [26]. The analysis of other synthetic cannabinoids lead to the assumption that in certain cases, the base peak is the molecular ion. Despite that, compounds in which α-cleavage is possible to occur present a base peak different than the molecular ion and resulting from the referred cleavage. In the case of these two compounds, one could think that they undergo α-cleavage, due to their base peaks not being the molecular ions. However, there is no prominent base peak, so this type of cleavage is most likely not occurring in these compounds, as it would lead to a very bulging peak. As seen in Figure 64, the presence of peaks *m/z* 144 ($C_9H_6NO^+$) and *m/z* 116 ($C_8H_6N^+$) indicate the presence of an indole moiety, as seen for the compounds discussed before [87].

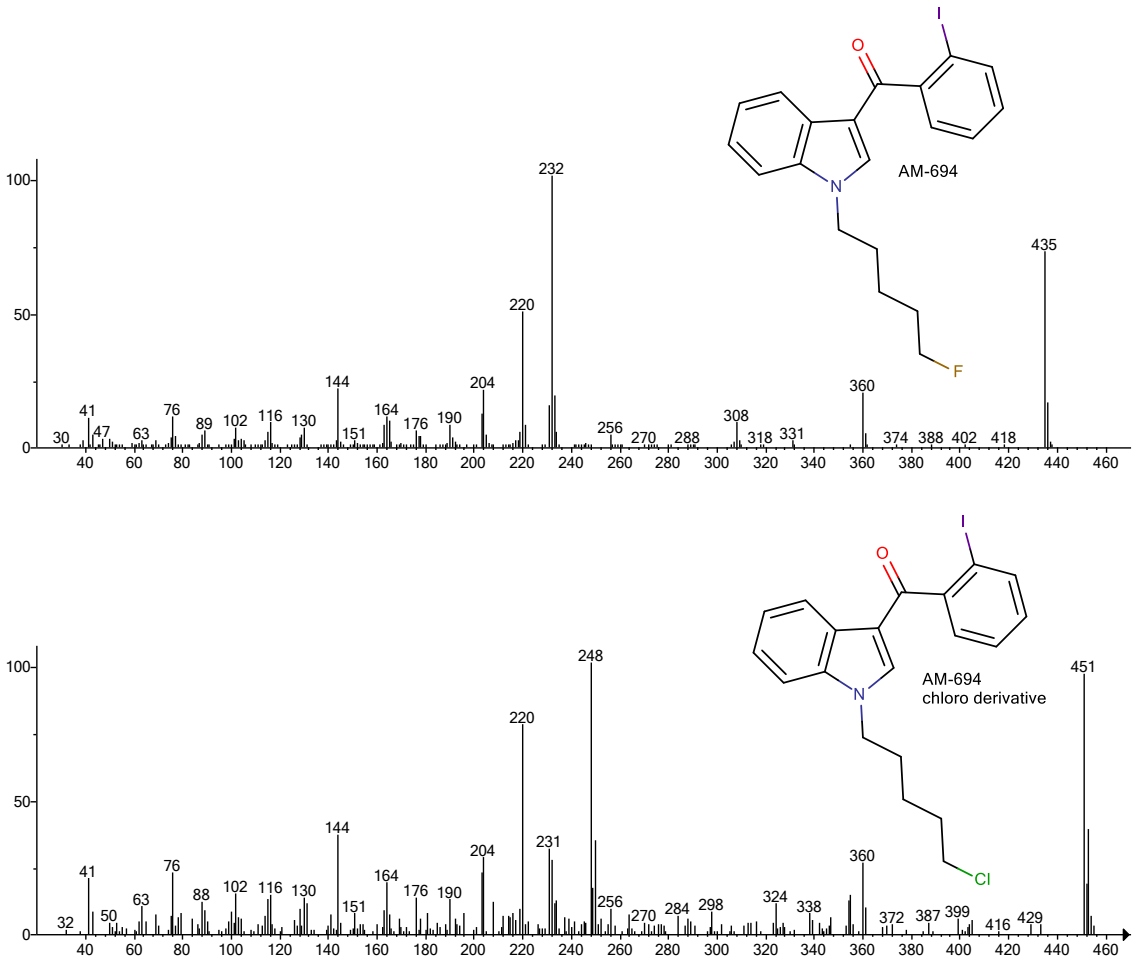


Figure 64 - EI-MS of two Benzoylindoles, AM-694 and AM-694 chloro derivative

However, there is no prominent base peak, so this type of cleavage is most likely not occurring in these compounds, as it would lead to a very bulging peak. As seen in Figure 64, the presence of peaks *m/z* 144 ($C_9H_6NO^+$) and *m/z* 116 ($C_8H_6N^+$) indicate the presence of an indole moiety, as seen for the compounds discussed before [87]. However, the absence of a peak at *m/z* 214 ($C_{14}H_{16}NO^+$) suggests other substitution on the indole group than a pentyl chain. Taking this into consideration, the base peak at *m/z* 232 has more 18Da than *m/z* 214. In the case of MAM2201 and JWH-122, this difference was seen and the presence of a fluor atom was observed. Hence, the base peak with *m/z* 232 could express the presence of a fluor atom and could result from the loss of the iodobenzyl moiety from the molecular ion. Also, the difference between molecular ion and base peak (203Da), suggests the presence of a iodo-substituted benzene ring (C_6H_4I = 203Da) (Figure 65) [88].

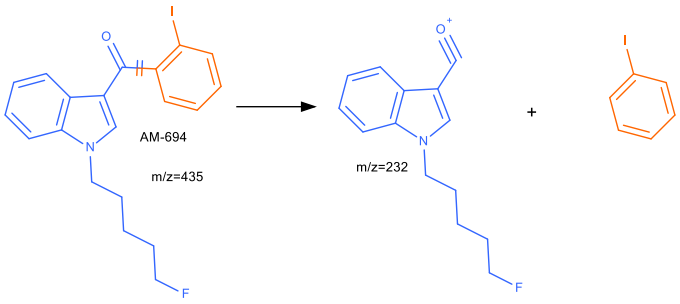


Figure 65 - Fragmentation of AM-694, forming the base at m/z 232

The same premise can be applied to the AM-694 chloro derivative. Its base peak is the peak at *m/z* 248, which has more 34Da than *m/z* 214. Thus, assuming that the substitution comprises a chlorine atom, this difference makes sense, has it will gain 35Da from the chlorine atom and lose and hydrogen atom (Figure 66).

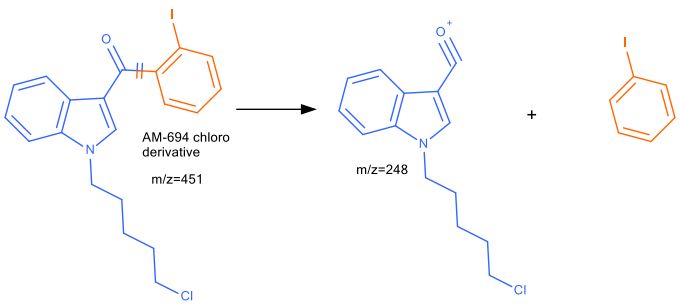


Figure 66 - Fragmentation of AM-694 chloro derivative, forming the base peak at m/z 248

The presence of a chlorine atom in the molecule can also be considered due to the presence of characteristic peaks that show the isotope ratio of the chlorine atom. Hence, the isotopic pattern of chlorine translates into two peaks, *m/z* 250 (*m/z* 248 + 2) and *m/z* 453 ([M^+] +2) with approximately 30% of the abundance of their parent ions. This phenomenon is characteristic of chlorine, since it represents the ^{37}Cl isotope, 30% abundant when compared to the ^{35}Cl isotope [35, 48].

As seen in the fragmentation of other compounds, the loss of the substitution in the indole group is plausible to occur. In the case of these two compounds, as the difference between them is in this substitution, the loss of it would lead to fragments of the same mass in the MS of both compounds. Hence, two peaks can be analysed, *m/z* 360 ($C_{16}H_{11}NIO^+$) and *m/z* 220 ($C_{15}H_{10}NO^+$) [89]. The formation of both fragments is proposed in Figure 67.

Taking into account the relative abundancies of both fragments, it could be suggested that the loss of the iodine atom is more plausible to occur, as peak *m/z* 220 ($C_{15}H_{10}NO^+$) is very abundant in both compounds.

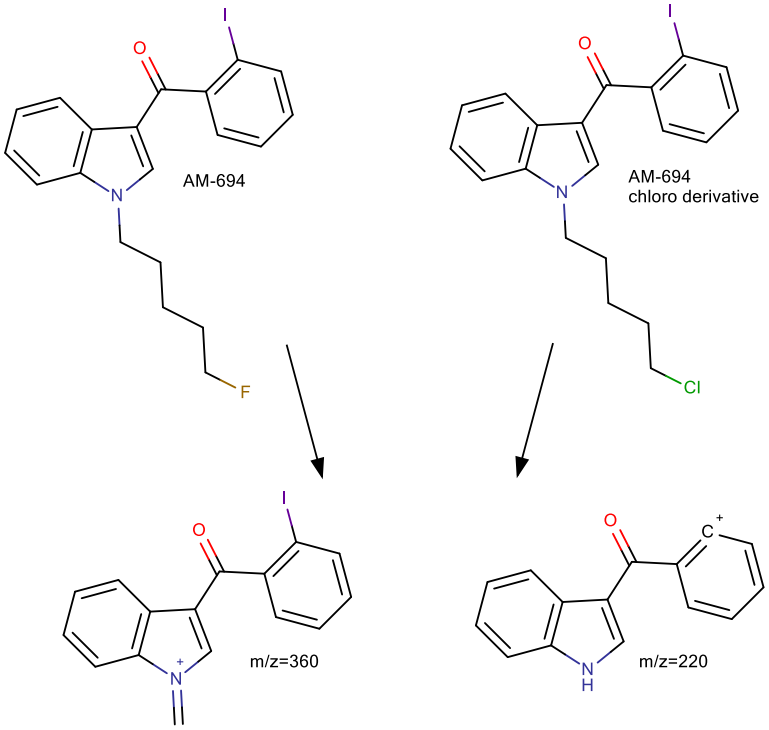


Figure 67 - Fragments m/z 360 and m/z 220, common to AM-694 and AM-694 chloro derivative

The final two compounds identified in this initial analysis by GC-EI-MS are AM1248 and AKB-48. According to this analysis, they have in common and adamantyl moiety on one of the sides of the carbonyl group. AM1248 was first reported to the EWS in 2012 by Germany [26] and, to the best of our knowledge, its identification was first reported by Uchiyama et al in 2012 [90]. The EI-MS of AM1248 reveals the presence of an abundant base peak at *m/z* 98. Considering the structure of the compound, α-cleavage is possible to occur between the piperidine group and the indole moiety, with charge being retained on the piperidine group, hence the peak at *m/z* 98 ($C_6H_{12}N^+$) (Figure 68).

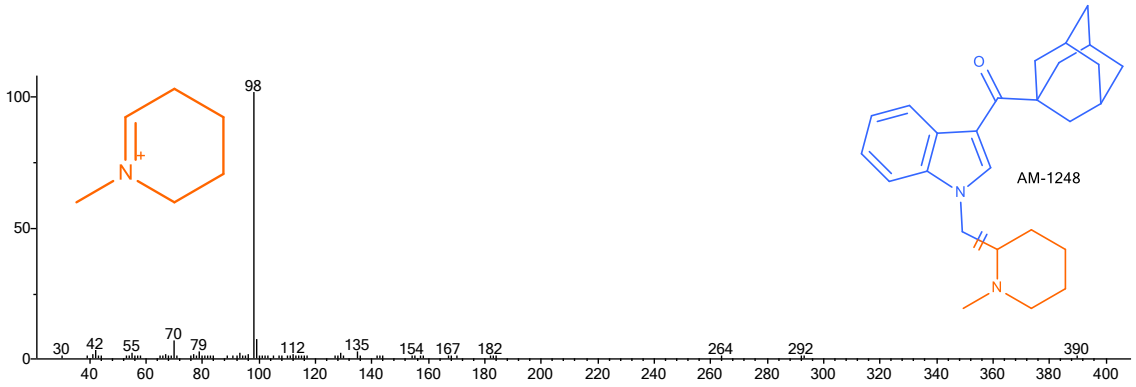


Figure 68 - EI-MS of AM1248

This is consistent with the literature as the same peak has been reported in studies on compounds with the presence of this group [90], [91], [92]. Due to the high intensity of the base peak, all the other peaks are not much abundant, but some of them are rather important in the analysis of the compound. The molecular ion is present [*m/z* 390 ($C_{26}H_{34}N_2O^+$)] as well as ions characteristic of the adamantyl group [*m/z* 135 ($C_{10}H_{15}^+$)] and also the ion formed via α-cleavage as well, but not as prominent as *m/z* 98, *m/z* 292 ($C_{20}H_{22}NO^+$) [90]. However, despite the fact that an indole moiety is present, the characteristic peaks mentioned before, *m/z* 214 ($C_{24}H_{16}NO^+$) and *m/z* 144 ($C_9H_6NO^+$), are not present, quite possibly due to the very low abundances of these peaks in the EI-MS of this compound. Again, other compounds with the piperidine group attached to an indole group do not show the characteristic peaks of an indole moiety [91].

AKB-48 is a synthetic cannabinoid that belongs to the family of the adamantoylindazoles, i.e., it has an indazole moiety, on the contrary of the other compounds discussed up to this point. This compound was first reported in the literature by Uchiyama et al in 2012 [90], under the name APINACA, and was reported in the EU in May 2012 [26]. Curiously, this compound got his current name, AKB-48, from a Japanese girl band, probably for marketing purposes [26]. Its EI-MS shows a great range of peaks with different abundancies (Figure 69). The presence of a carboxamide as the linking group between the two moieties gives rise to its base peak, resultant from cleavage of the C-N bond [48] and characteristic of a pentyl-substituted indazole group, *m/z* 215 ($C_{13}H_{15}N_2O^+$). The fact that this peak is characteristic of an indazole group can

be derived from the characteristic peak of an indole, *m/z* 214 ($C_{14}H_{16}NO^+$). The difference between indazole and indole groups is the addition of a nitrogen atom (14Da) and the loss of a carbon and hydrogen atoms (13Da).

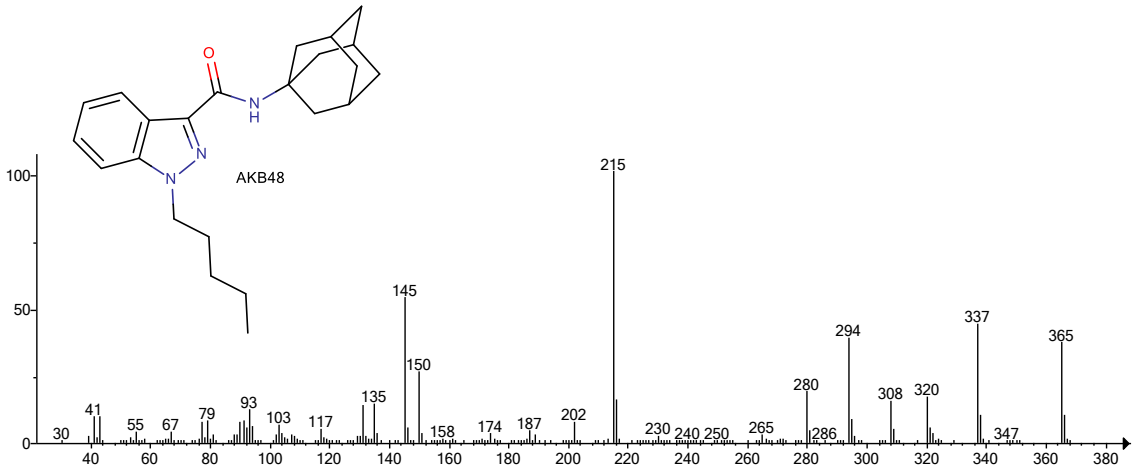


Figure 69 - EI-MS of AKB-48

Alike, the presence of fragments *m/z* 145 ($C_8H_5N_2O^+$) and *m/z* 117 ($C_7H_5N_2^+$) are characteristic of an indazole moiety. The presence of the adamantyl group and the carboxamide linker can be assessed by peaks *m/z* 135 ($C_{10}H_{15}^+$), as seen for AM1248 (Figure 70) and *m/z* 150 ($C_{10}H_{16}N^+$), resulting from cleavage of the C-N bond on the carboxamide group, with charge retained on the nitrogen atom attached to the adamantyl group (Figure 70).

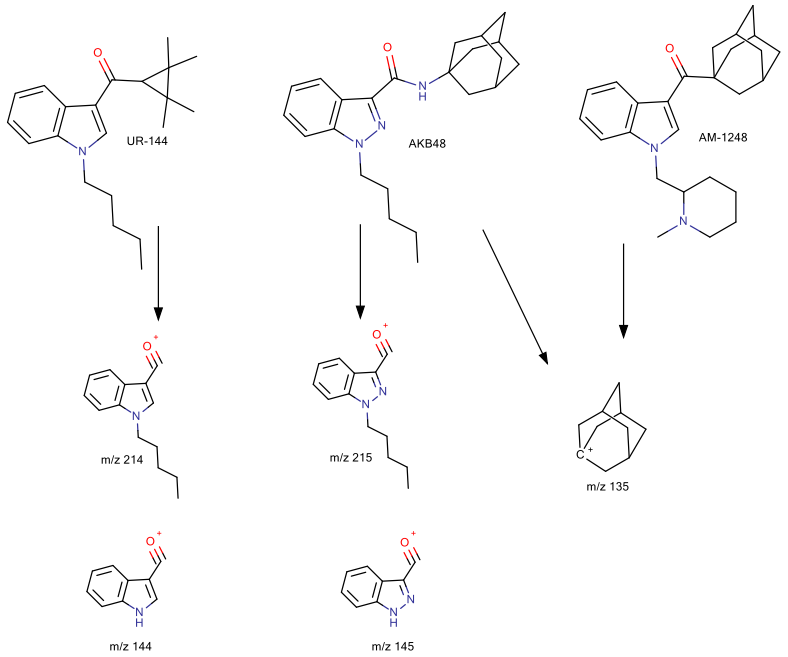


Figure 70 - Indazole and Adamantyl fragments of AKB48: Comparison with fragmentation of UR-144 nad AM1248

This initial GC-EI-MS analysis of herbal incenses permitted the identification of 10 different synthetic cannabinoids (Figure 71) in 23 different herbal incense products. This analysis served as an indicator of the possible content of the packages and was useful as a screening tool for the second part of this project. The EI-MS spectra analysis allowed the identification of characteristic fragments that could be used in further analysis, especially if a new compound emerges.

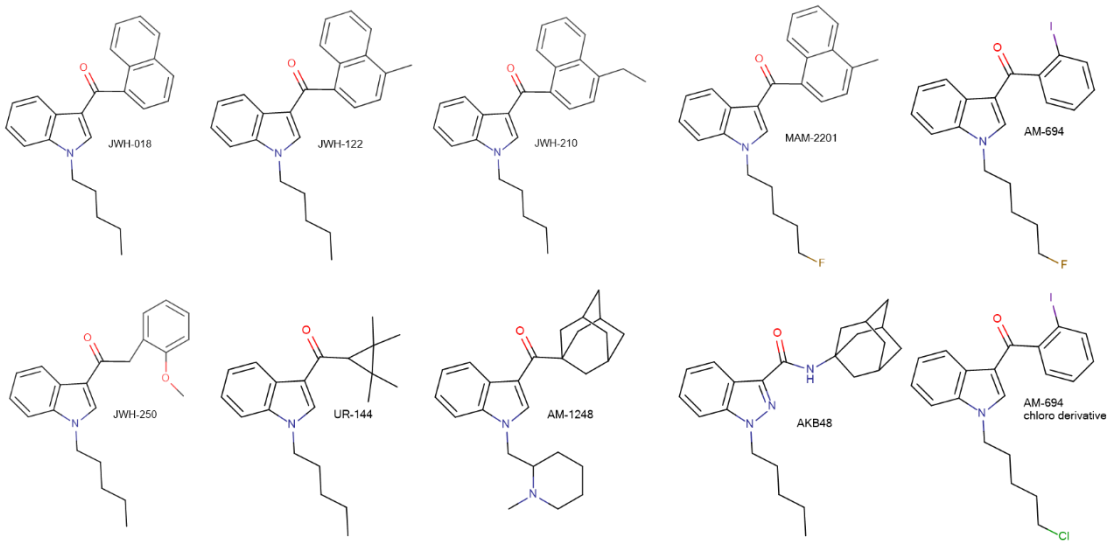


Figure 71 - Synthetic Cannabinoids detected in the Preliminary GC-EI-MS analysis

3.2.2. Isolation of Compounds from Herbal Incenses

After the preliminary analysis, new samples were chosen according to the possible contents verified by the first analysis, in order to characterise the compounds by NMR in order to add synthetic cannabinoids to the in-house EI-MS library. Due to the complexity of the samples, i.e., due to the fact that they are present in an herbal matrix, an isolation process by liquid chromatography was first employed, in order to have the compounds in a pure form to be characterised by NMR, as to confirm the presence of the compounds previously analysed by GC-MS. Therefore, based on the results shown in Table 14, samples of Esfinge, Magic, Maya2012, Spike 99, Spliff and Radioactive were chosen in order to isolate all the synthetic cannabinoids previously detected by GC-EI-MS in herbal incenses (Figure 71). At least two packages of each product were chosen and were firstly analysed by GC-EI-MS to check if the composition was the same (Table 15).

Table 15 - Composition of the analyses on herbal incenses with the same brand name

Product	First GC-MS	Second GC-MS	
		Sample	***Result***
Esfinge	JWH-210	*1*	JWH-210
		2	JWH-210
Magic	JWH-018	*1*	JWH-018
		2	JWH-018 MAM2201
Maya2012	UR-144 AKB-48	*1*	UR-144 AKB-48
		2	XLR-11 AKB-48
Spliff	JWH-250 JWH-122	*1*	JWH-250 JWH-122
		2	JWH-250
		3	-
		4	-
		5	-
Spike99	5-MeO-DALT AM694 JWH-122 Cl-AM694 AM1248	*1* *2* *3* *4* *5*	5-MeO-DALT AM694 JWH-122 Cl-AM694 AM1248
Radioactive	5-MeO-DALT AM694 JWH-122 Cl-AM694 AM1248	*1* *2* *3* *4* *5*	5-MeO-DALT AM694 JWH-122 Cl-AM694 AM1248

As seen in Table 15, different packages of the same brand name revealed the presence of different compounds, thus indicating a possible variability within the products. From the new detected compounds, one has not been detected in the previous analysis.

XLR-11, or 5F-UR-144, is the fluor derivative of UR-144 and was first reported to the EWS in February 2012, by Latvia [26]. To the best of our knowledge, the first time that the occurrence of XLR-11 in herbal incenses was reported in the literature was in 2013 by Choi et al [81]. Figure 72 shows the EI-MS spectrum of XLR-11.

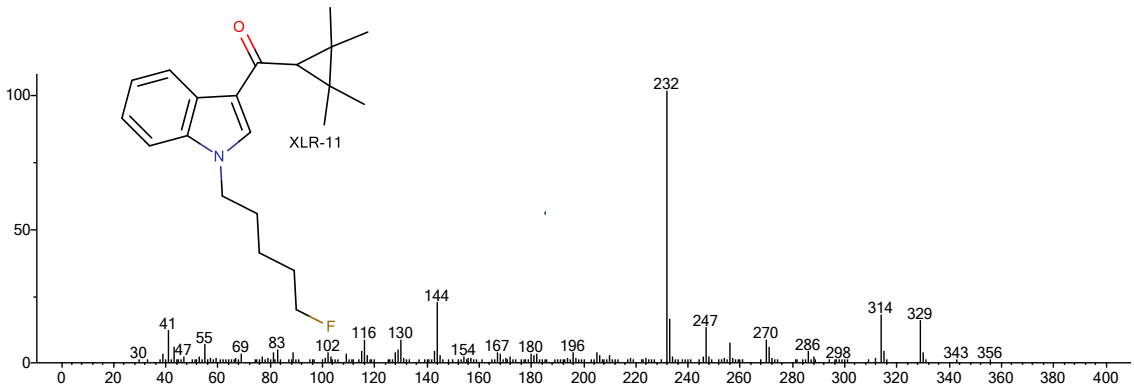
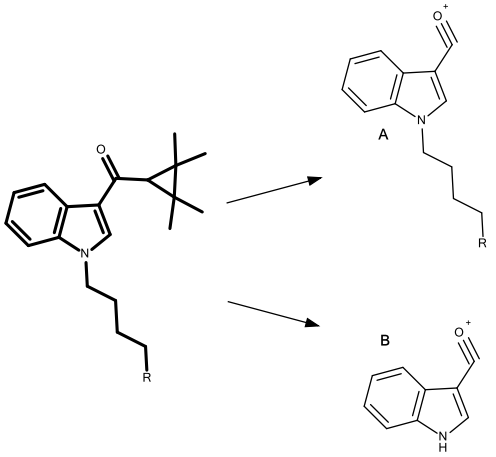


Figure 72 - EI-MS of XLR-11, the 5-pentyl fluorinated analogue of UR-144

As seen in the comparison between JWH-122 and MAM-2201, the comparison of fragmentations between XLR-11 and UR-144 can facilitate the identification of this compound (Figure 73).



	UR-144
	R=CH_3; M.W. = 311
A	214
B	144
	XLR-111
	R=CH_2F; M.W. = 329
A	232
B	144

Figure 73 - Comparison of Fragments of UR-144 and XLR-11

As seen in other cases, the fluor substitution on the aliphatic chain increases all the correspondent peaks in 18Da. Therefore, the characteristic peaks of the indole moiety where fluor is present are the base peak *m/z* 232 ($C_{14}H_{15}FNO^+$), result from α-cleavage and the base peak *m/z* 329 ($C_{21}H_{28}FNO^+$).

Again, the presence of possible characteristic peaks of the tetramethyl-substituted cyclopropane moiety are not present, due to its facile thermal degradation [83]. Nonetheless, as observed in UR-144, this compound also reveals the presence of its cyclopropyl rearrangement product, therefore allowing for a more correct identification of the compound.

All the characteristic peaks from the EI-MS of the rearrangement product are the same as the ones observed for XLR-11 [81]. Anyway, as observed in UR-144, the cyclopropyl rearrangement product has a second peak, adjacent to the base peak, with a 15Da increase, leading to fragment *m/z* 247 ($C_{15}H_{18}FNO^+$), which results from rearrangement on the cyclopropyl, followed by cleavage [81] (Figure 74).

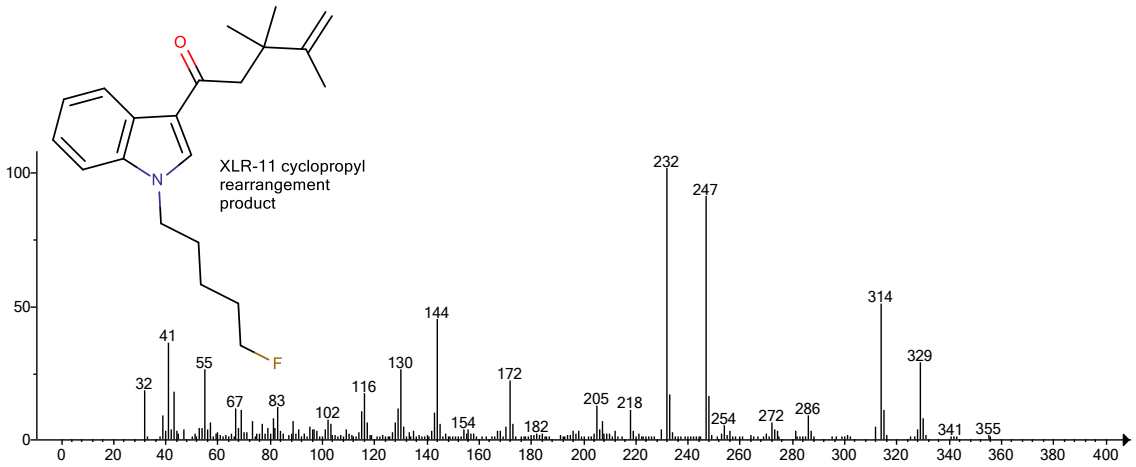


Figure 74 - EI-MS of the cyclopropyl rearrangement product of XLR-11

After all the compounds were identified by EI-MS, methanolic extraction of the herbal incense products was performed in order to isolate the synthetic cannabinoids of interest by liquid chromatography. The purified NPS were then identified by NMR spectroscopy.

3.2.3. NMR Analyses of Isolated Synthetic Cannabinoids

Three synthetic cannabinoids were isolated from their matrix by liquid chromatography and the purified compounds were analysed by NMR. From the methanolic extract of herbal incense product Magic (lote '2012 43XMGO', smartshop H) were isolated Compound 1 and Compound **3**, and from product Esfinge (lote 2012 43X28M, smartshop H), Compound **2**. All three synthetic cannabinoids were identified by GC-EI-MS analysis as JWH-018 (Compound **1**), JWH-122 (Compound **3**) and JWH-210 (Compound **2**).

Analysis of ^{1}H NMR spectrum of Compound **1** in $CDCl_3$ revealed the presence of 23 protons: a methyl signal at δ0.86 (3H, t, *J* = 6.80Hz); three methylene signals at δ1.25 (2H, m, overlapped), δ1.29 (2H, m, overlapped) and δ1.81 (2H, m, *J* = 7.20Hz); a methylene signal connected to a nitrogen atom at

δ4.07 (2H, t, *J* = 7.20Hz) and 12 aromatic signals at δ7.36 (1H, s), δ7.37 (1H, m, overlapped), δ7.37 (1H, m, overlapped), δ 7.39 (1H, overlapped), δ7.47 (1H, m, overlapped), δ7.51 (1H, m, overlapped), δ7.53 (1H, m, overlapped), δ7.67 (1H, d, *J* = 6.40Hz), δ 7.92 (1H, d, *J* = 7.60Hz), δ 7.97 (1H, d, *J* = 8.00Hz), δ 8.20 (1H, d, *J* = 8.00Hz) and δ 8.51 (1H, m, overlapped).

^{13}C APT NMR spectrum revealed the presence of 24 carbons, suggesting a carbonyl carbon at δ 192.00, 6 aromatic quaternary carbons (δ117.46, δ126.94, δ130.74, δ133.68, δ136.98 and δ139.04), 12 aromatic methine carbons (δ109.96, δ122.81, δ122.87, δ123.56, δ124.52, δ125.80, δ125.95, δ126.25, δ126.71, δ128.12, δ129.92 and δ137.95), 1 methylene with a nitrogenated carbon (δ47.13), 3 methylene carbons (δ22.13, δ28.85 and δ29.44) and 1 methyl carbon at δ13.84.

The assignment off all signals was permitted by using 1D and 2D correlation techniques, as seen in Figure 75 and Table 16.

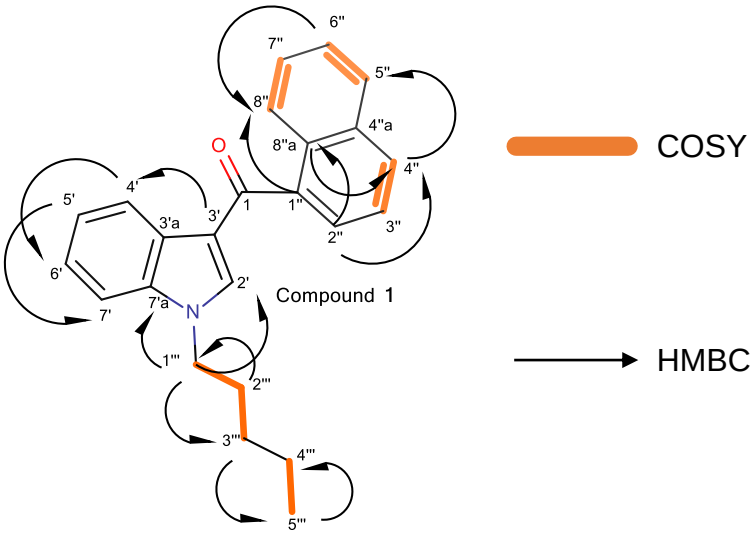


*Figure 75 – Key COSY and HMBC correlations in Compound **1***

Attribution of the signals for Compound **1** started with the aliphatic signals, from the terminal methyl group (δ_H0.86t/ δ_C13.84) to the *N*-CH_2 group (δ_H4.07t/ δ_C47.13). Using the two-dimensional (2D) correlation NMR techniques COSY (homonuclear 1H-1H) and HMBC (heteronuclear 1H-13C), it was possible to determine the position of the others 3 methylene groups (δ_H1.25m/

δ_C28.85; δ_H1.29m/ δ_C22.13; δ_H1.82m/ δ_C29.44) on the aliphatic chain (Figure 76).

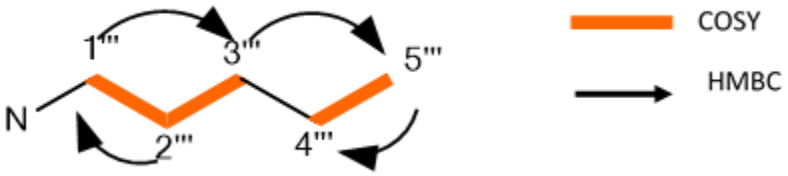


Figure 76 - Selected correlations for the aliphatic chain of Compound 1 by COSY and HMBC

The COSY correlations (between the methylene at δ_H1.29 and the terminal methyl at δ_H0.86 ((C5'''), and between the methylene at δ_H1.81 and *N*-CH_2 at δ_H 4.07 (C1''')), and the HMBC correlations (between the proton signal of *N*-CH_2 and the carbons δ_C28.85/δ_C 29.44, and between the terminal methyl and the carbons δ_C28.82/ δ_C22.13), allowed for the correct attribution of signals at 2''', 3''' and 4''' positions (Figure 76). The HMBC correlations of HC1''' with the methine carbon δ_C137.95 and the quaternary carbon δ_C139.98 enabled the assignment of the NMR signals at positons 2' and 7'a, respectively. The aromatic proton δ_H7.67d (125.80) was assign to C2'' by its HMBC correlation with the carbonyl C1.

The C4'' (δ_C 129.92) was confirmed with HMBC correlations with 5'', 8'a and 2''. H5'' also showed a COSY correlation with H6'', which revealed a HMBC correlation with C8''. H8'' showed a COSY correlation with the missing aromatic proton, H7'' and HMBC correlation with a quaternary carbon (δ_C 133.68), assigned as 4''a.

At this point, there are 3 quaternary carbons left to assign. HMBC allowed for the attribution of C1'', due to a correlation with H8''. C3' and C3'a with value of δ_C117.46 and δ_C126.94 showed HMBC correlations with aromatic protons of the indole moiety, and comparison with the literature of other indole cannabinoids revealed the expected values for this carbons (117.45-117.6; 127-127) leading to their attribution [93, 79, 70, 80]. The HMBC correlations of C3' with the indole proton at 8.51m ppm means that it must be the H4'. The other aromatics carbons where assigned by HMBC correlations between C4' and H6' and between C5' and H7' (also confirmed by existing literature) [93, 79, 70, 80].

Table 16 - ^{1}H and ^{13}C NMR data of Compound 1 and JWH-018 (from literature)

Position	**JWH-018** [93]		**Compound 1**	
	^{13}C	**HSQC**	**^{13}C**	**HSQC**
1	192.0	-	192.00	-
2'	137.9	7.33s	137.95	7.36s, 1H
3'	117.5	-	117.46	-
3'a	127.0	-	126.94	-
4'	122.9	8.47m	122.87	8.51m, 1H, overlapped
5'	122.8	7.35m	122.81	7.37m, 1H, overlapped
6'	123.6	7.35m	123.56	7.37m, 1H, overlapped
7'	110.0	7.38m	109.96	7.39m, 1H, overlapped
7'a	137.0	-	136.98	-
1''	139.1	-	139.04	-
2''	125.8	7.64dd	125.80	7.67d, 1H *J* = 6.40Hz
3''	124.5	7.51dd	124.52	7.53m, 1H, overlapped
4''	129.9	7.95brd	129.92	7.97d, 1H, *J* = 8.00Hz
4''a	133.7	-	133.68	-
5''	128.1	7.90brd	128.12	7.92d, 1H, *J* = 7.60Hz
6''	126.3	7.50td	126.25	7.51m, 1H, overlapped
7''	126.7	7.45ddd	126.71	7.47m, 1H, overlapped
8''	126.0	8.17brd	125.95	8.20d, 1H, *J* = 8.00Hz
8''a	130.8	-	130.74	-
1'''	47.2	4.05t	47.13	4.07t, 2H, *J* = 7.20Hz
2'''	29.5	1.79quint	29.44	1.81m, 2H, *J* = 7.20Hz
3'''	28.9	1.24m	28.85	1.25m, 2H, overlapped
4'''	22.2	1.28m	22.13	1.29m, 2H, overlapped
5'''	13.8	0.83t	13.84	0.86t, 3H, *J* = 6.80Hz

The signal attribution of unknown compound **1** and its comparison with a published attribution of JWH-018 can be seen in Table 16. Therefore, Compound **1** was elucidated as JWH-018, as predicted by the EI-MS analysis.

As seen in the MS analysis, Compounds **2** and **3** could respectively be JWH-210 and JWH-122. Structurally, they differ from JWH-018 by the presence of an ethyl group or a methyl group, respectively, on the naphthalene moiety. Hence, it should be simple to assign to elucidate the structures of Compound **2** and **3** by comparing their NMR spectra with the spectra of Compound **1**.

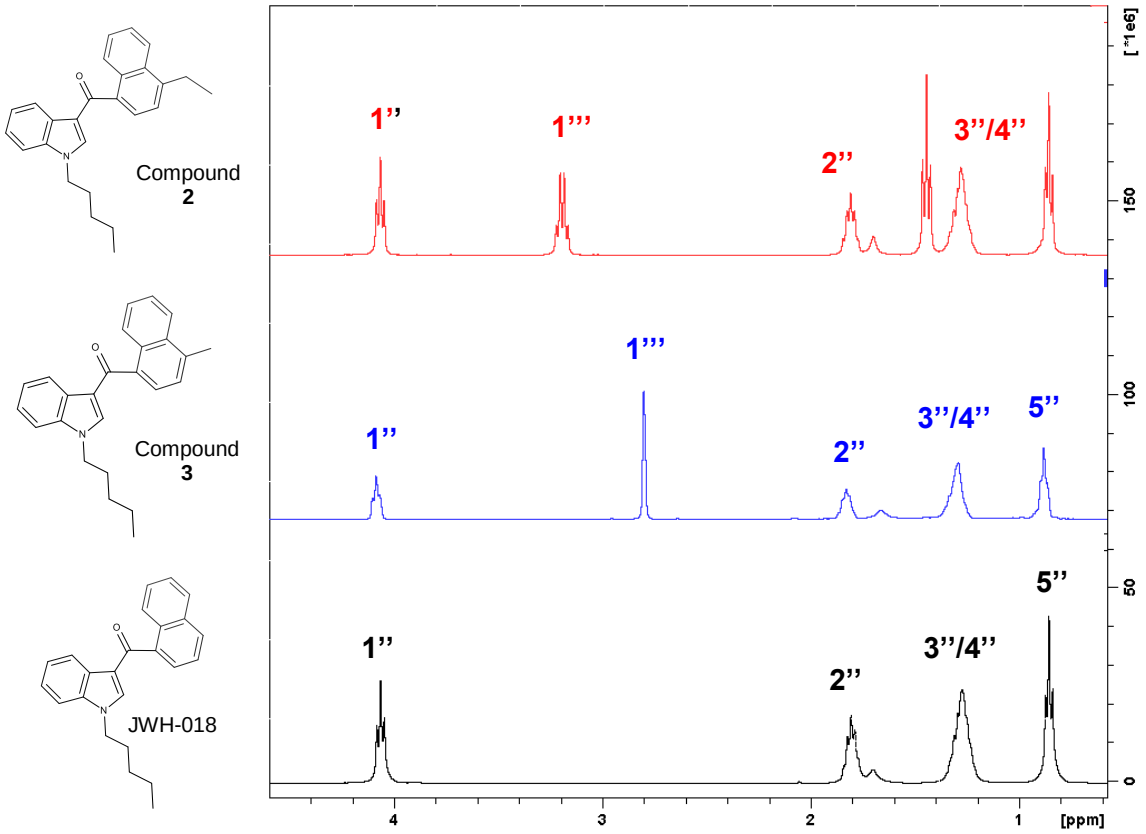


*Figure 77 - Comparison of High Field 1H NMR Spectra of JWH-018 (black), Compound **3** (blue) and Compound **2** (red)*

The ^{1}H NMR spectra of Compound **2** and Compound **3** revealed the presence of extra alkyl signals, two for Compound **2** (3.19q; 2H; *J*=7.20Hz and 0.86t; 3H; *J*=7.20Hz, characteristic of an ethyl group) and one for Compound **3** (2.78s; 3H characteristic of a methyl group) (Figure 77). Also, in the low field region (or aromatic region) is possible to see the influence of the alkyl groups on the chemical shifts of the aromatic protons (Figure 78).

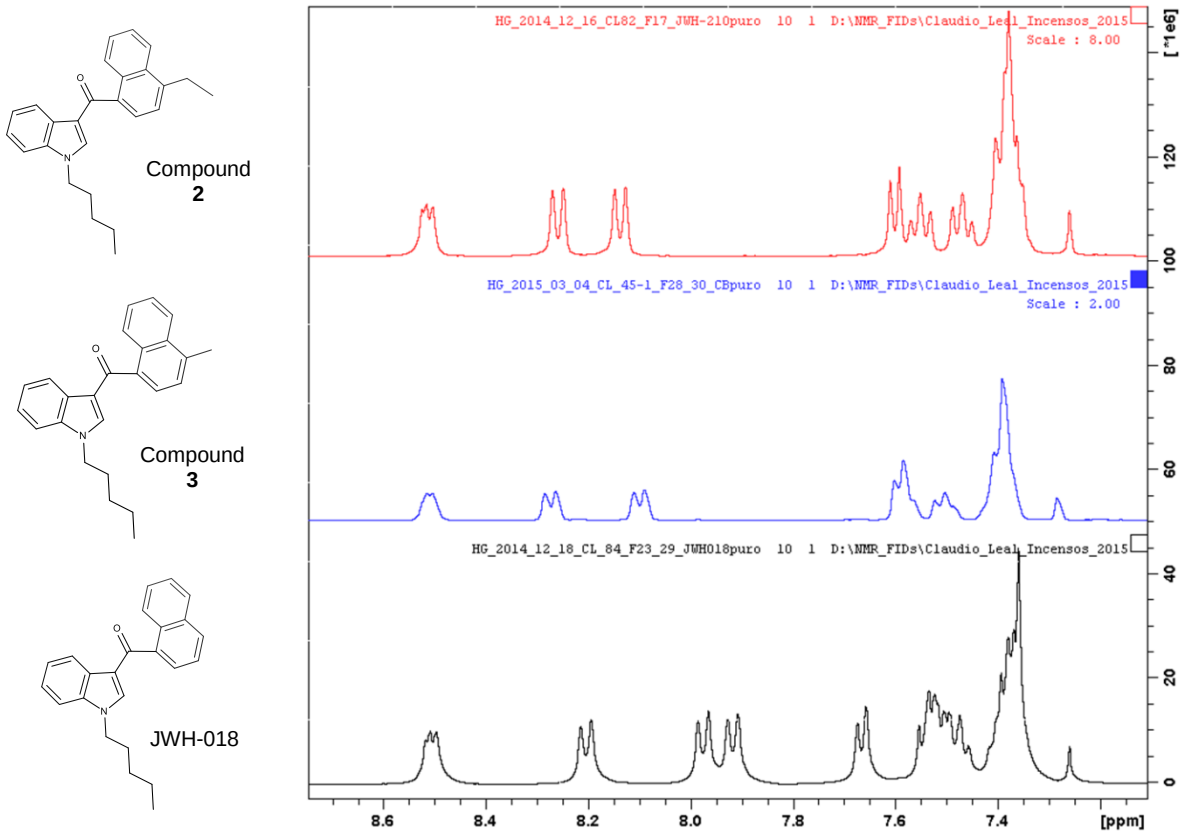


*Figure 78 - Comparison of Low Field 1H NMR Spectra of JWH-018 (black), Compound **3** (blue) and Compound **2** (red)*

The ^{13}C NMR of Compound **2** showed the presence of an extra methyl carbon (δ_C 14.90) and an extra methylene carbon (δ_C 26.17) and Compound **3** revealed the presence of an extra methyl carbon (δ_C 19.79). All this is in accordance with the possible matches of JWH-210 and JWH-018 for Compounds **2** and **3**, respectively [75, 74].

Table 17 shows the comparison of attributed signals of all three compounds and respective literature on JWH-018, JWH-210 and JWH-122.

*Table 17 - NMR Assignments of Compounds **1**, **2** and **3** and respective comparison with the literature*

Position	**JWH-210** [70]		Compound 2		**JWH-122** [70]		Compound 3		**JWH-018** [93]		Compound 1	
	^{13}C	^{1}H	^{13}C	HSQC	^{13}C	^{1}H	^{13}C	HSQC	^{13}C	^{1}H	^{13}C	HSQC
1	192.3	-	192.30	-	192.3	-	192.00	-	192.0	-	192.00	-
2`	137.5	7.37s	137.90	7.38m	137.9	7.37brs	137.79	7.38s	137.9	7.33s	137.95	7.36s
3`	117.7	-	117.60	-	117.8	-	117.68	-	117.5	-	117.46	
3a`	127.1	-	127.01	-	127.2	-	127.04	-	127.0	-	126.94	
4`	123.0	8.49m	122.9	8.49	122.9	8.49m	122.74	8.49m	122.9	8.47m	122.87	8.51m
5`	122.7	7.35m	122.74	7.35	123.1	7.36m	122.92	7.37m	122.8	7.35m	122.81	7.37m
6`	123.8	7.35m	123.49	7.35	123.6	7.36m	123.49	7.37m	123.6	7.35m	123.56	7.37m
7`	109.9	7.39m	109.9	7.40	110.0	7.39m	109.91	7.39m	110.0	7.38m	109.96	7.39m
7a`	137.0	-	137.0	-	137.1	-	137.01	-	137.0	-	136.98	-
1”	137.8	-	137.42	-	137.7	-	137.56	-	139.1	-	139.04	
2”	125.9	7.59brd	125.91	7.60d	125.9	7.57brd	125.80	7.56d	125.8	7.64dd	125.80	7.67d
3”	123.5	7.38m	123.49	7.39	125.4	7.38m	125.25	7.38m	124.5	7.51dd	124.52	7.53m
4”	142.5	-	142.5	-	136.7	-	136.62	-	129.9	7.95brd	129.92	7.97d
4a”	132.0	-	132.0	-	132.9	-	132.80	-	133.7	-	133.68	
5”	125.8	8.13d	123.8	8.14d	124.3	8.08brd	124.18	8.08d	128.1	7.90brd	128.12	7.92d
6”	126.2	7.54td	126.06	7.55t	126.2	7.56td	126.11		126.3	7.50td	126.25	7.51m
7”	126.8	7.46td	126.21	7.47t	126.5	7.48ddd	126.36	7.49m	126.7	7.45ddd	126.71	7.47m
8”	126.0	8.25d	126.75	8.26d	126.7	8.25brd	126.61	8.25d	126.0	8.17brd	125.95	8.20d
8a”	131.2	-	131.10	-	131.0	-	130.89	-	130.8	-	130.74	
1”’	47.1	4.07t	47.12	4.07t	47.2	4.07t	47.12	4.06t	47.2	4.05t	47.13	4.07t
2”’	29.5	1.80quint	29.47	1.81m	29.6	1.82quint	29.46	1.81m	29.5	1.79quint	29.44	1.81m
3”’	28.9	1.26m	28.87	1.26m	29.1	1.32m	28.89	1.27m	28.9	1.24m	28.85	1.25m
4”’	22.2	1.28m	22.15	1.29m	22.3	1.27m	22.15	1.31m	22.2	1.28m	22.13	1.29m
5”’	13.8	0.85t	13.81	0.86t	14.0	0.86t	13.83	0.86t	13.8	0.83t	13.84	0.86t
1””	26.2	3.18quartet	26.17	3.19q	19.9	2.79s	19.79	2.78s	-	-	-	-
2””	14.9	1.44t	14.90	1.45t	-	-	-	-	-	-	-	-

The preliminary GC-EI-MS analysis of 33 herbal incenses allowed the detection and identification of 10 synthetic cannabinoids (JWH-018, JWH-122, JWH-210, JWH-250, MAM2201, AKB48, UR-144, AM694, AM694-chloro derivative and AM1248). A second GC-EI-MS analysis of products that contained only one or two synthetic cannabinoids was performed, as to verify the composition of the packages and also check for the possible existence of different compounds. Through this, it was possible to identify MAM2201 in a different package and XLR-11, a new compound that was not detected during the preliminary analysis. From the 11 identified cannabinoids, only 7 were until now isolated by our group. The 3 naphthoylindole cannabinoids, JWH-018, JWH-122 and JWH-210 were isolated from Esfinge and Magic products in the scope of this project. The 4 synthetic cannabinoids (AKB48, UR-144, XLR-11 and MAM2201) detected in herbal incense samples were purified and identified trough NMR spectroscopy by Carlos Branco, within his project, and by Christophe Gonçalves, within his ERASMUS internship. The analysis of herbal incenses and subsequent isolation and characterisation of those compounds by NMR allowed the unequivocal identification of the 7 synthetic cannabinoids as predicted by the EI-MS analysis.

All these seven Isolated and characterised compounds were then analysed by GC-EI-MS by the method recommended by the UNODC, in order to create an in-house EI-MS library of synthetic cannabinoids. Table 18 shows the GC-EI-MS characteristics of the seven purified compounds.

Table 18 - In-house EI-MS library of 7 synthetic cannabinoids

Compound	R_t (min)	Base Peak	Other EI Fragmentations
JWH-210	16.2	369 [M^+]	312, 298, 214, 183, 144
JWH-018	14.1	341 [M^+]	284, 270, 214, 155, 144, 127
JWH-122	15.6	355 [M^+]	298, 284, 214, 169, 144, 141
MAM2201	17.2	373 [M^+]	298, 284, 232, 144, 141, 115
AKB-48	13.6	215	365 [M^+], 308, 294, 187, 135
XLR-11	8.3	232	329 [M^+], 314, 144
UR-144	7.0	214	311 [M^+], 144

3.2.4. SGT-25: A novel cannabinoid in a seized sample

As seen in the case of 4F-PBP, the collaboration between FCUL and LPC/PJ also allows the assistance of the faculty in the identification of novel substances that may arise within the casework of the toxicology sector at LPC/PJ. A yellowish oil was seized and in its packaging, the following label was present: “1(5-fluoropentyl)-N-2-phenylpropan-2-yl)indazole-3-carboxamide”. This label suggests a chemical structure, as see in Figure 80.

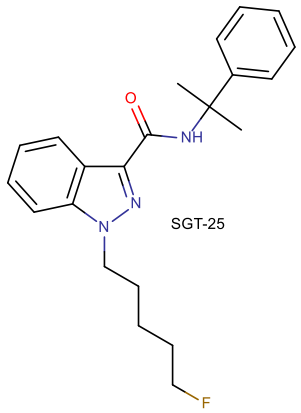


Figure 79 - Chemical structure of SGT-25

A sample of the yellowish oil was analysed by GC-MS and its analysis revealed the presence of an unknown compound, even after comparison with the existing MS libraries, like SWGDRUG, ENFSI or Cayman. This is due to the fact that this compound was just reported for the first time in 2014.The sample was analysed by GC-EI-MS and the resulting MS is shown in Figure 80.

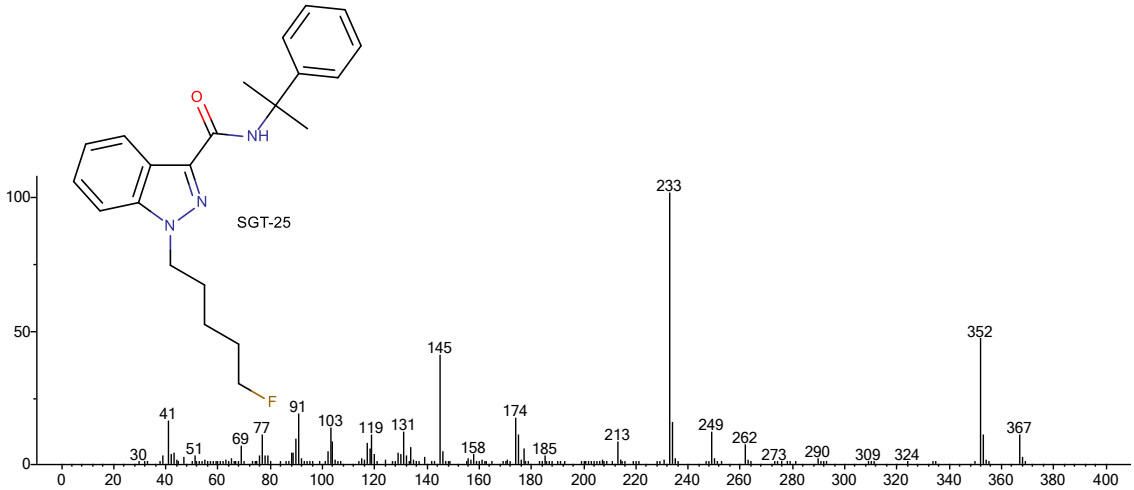


Figure 80 - EI-MS of SGT-25

As expected considering the structure of the compound, its base peak is not its molecular ion, as the compound undergoes α-cleavage, originating a base peak at *m/z* 233 ($C_{13}H_{14}FN_2O^+$). Considering its indazole moiety, comparison of this result can be made with AKB-48, another indazole-type synthetic cannabinoid discussed earlier. The fluor substitution on the indazole can be assessed by the base peak of SGT-25. In AKB48, with a pentyl-substitution on the indazole group, the base peak is *m/z* 215 ($C_{13}H_{15}N_2O^+$). As discussed earlier, the presence of a fluor atom will increase a fragment mass in 18Da. Accordingly, the difference of the base peaks of the two compounds is 18Da (*m/z* 232 – *m/z* 215 = 18Da), thus confirming it fluor-substitution. Peak at *m/z* 145 also indicates the presence of an indazole group, again, as seen for AKB48 (Figure 81).

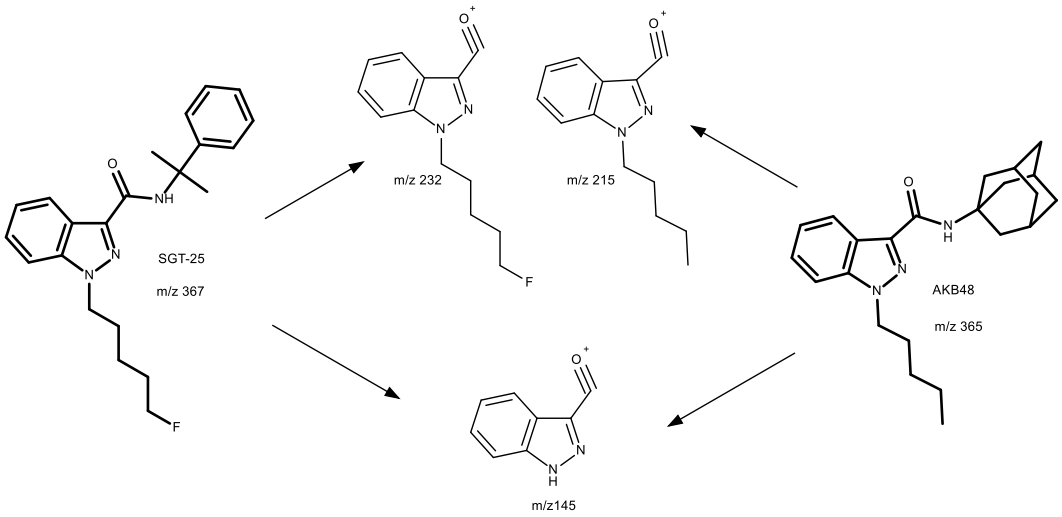


Figure 81 - EI-MS fragmentations of the Indazole moiety of SGT-25 and AKB48

The presence of a benzyl group on the other side of the carboxamide group is estimated by the presence of fragment *m/z* 91 ($C_7H_7^+$), the tropiylium ion characteristic of phenylalkanes, as seen in the case of 4-MEC [32, 35], However, the substitution of a cumyl group can be assessed by other peaks, at *m/z* 119 ($C_9H_{11}^+$), resulting from cleavage from the carboxamide group Figure 82).

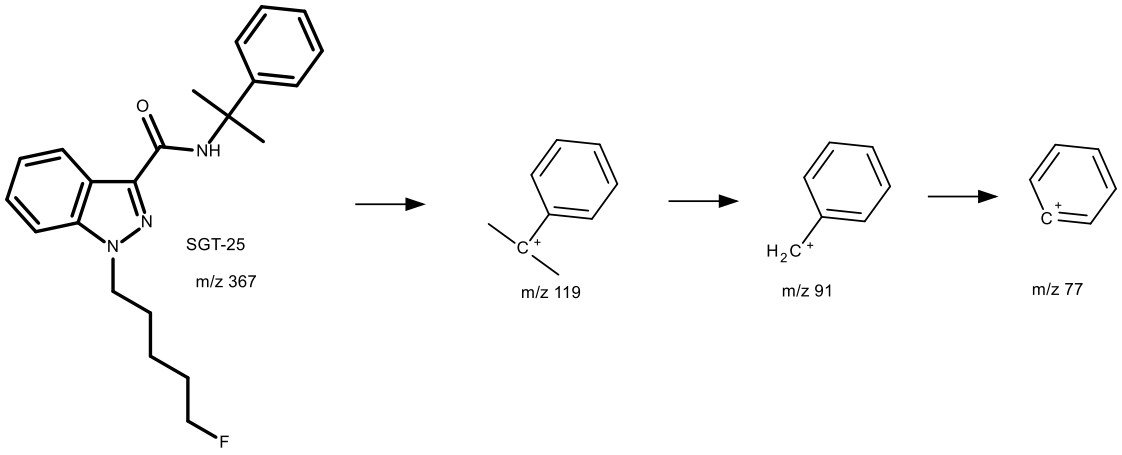


Figure 82 - EI-MS fragmentation of SGT-25 on the cumyl moiety

After analysis by GC-MS, the sample was analysed at FCUL by NMR in $CDCl_3$.

Analysis of the ^{1}H NMR spectrum of SGT-25 in $CDCl_3$ revealed the presence of 26 protons: two methyl signals at δ1.87 (6H, s); three methylene signals at δ1.48 (2H, m, *J*=7.6Hz), δ1.75 (2H, dq) and δ2.01 (2H, m, *J*=7.6Hz); a methylene signal connected to a nitrogen atom at δ4.40 (2H, t, *J* = 7.2Hz); a methylene signal geminal to a fluor atom at δ4.43 (2H, dt, *J*=47.2Hz) and seven aromatic protons at δ7.21 (1H, m, overlapped), δ7.23 (1H, t, overlapped), δ7.34 (2H, t, *J*=7.6Hz), δ7.39 (1H, m, overlapped), δ7.39 (1H, m, overlapped), δ7.52 (2H, d, *J*=8.0Hz) and δ8.34 (1H, d, *J*=8.0Hz).

^{13}C NMR spectrum revealed the presence of 22 carbons, suggesting a carbonyl carbon at δ161.00, four aromatic quaternary carbons at δ122.76, δ137.80, δ140.81 and δ147.17; one quaternary carbon coupled to two methylene groups at δ55.75; nine aromatic carbons at δ108.95, δ122.38, δ123.18, δ124.80 (2 carbons), δ126.53, δ126.68, δ128.38 (2 carbons); one methylene carbon attach to a nitrogen atom at δ49.04; three methylene carbons at δ22.65 (d, *J*=5.0Hz), δ29.31 and δ29.86 (d, *J*=19.72Hz); one methylene carbon coupled to a fluor atom at δ83.66 (d, *J*=164.80) and two methyl carbons at δ29.56.

Assignment of each carbon to the corresponding protons was possible with HSQC correlations (Table 19)

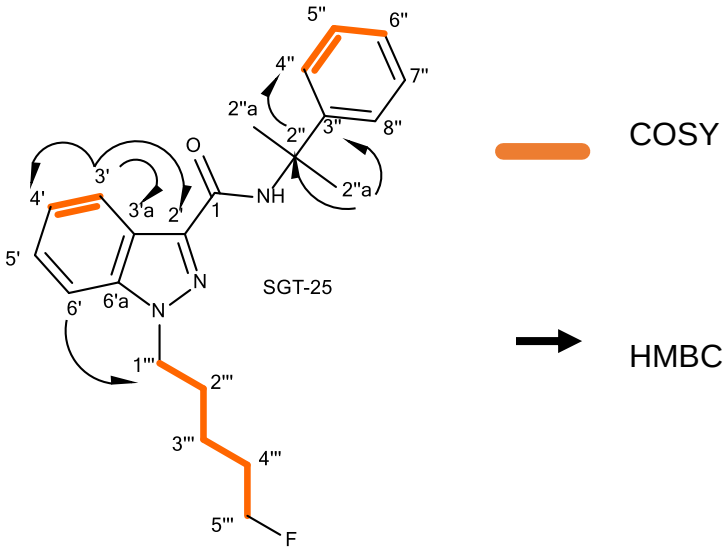


Figure 83 - Key COSY and HMBC correlations for SGT-25

Attribution of the signals for SGT-25 started with the methyl groups at δ_H1.87s which revealed the presence of 6 protons, correspondent to the two equivalent methyl groups at C2''a. HMBC correlation of this proton signal with δ_C55.75 and δ_C 147.17 allowed the assignment of two quaternary carbons 2'' and 3'', respectively. HMBC correlations of C2'' with the aromatic protons δ_H7.52d permitted the attribution of the chemical equivalent aromatic signals at positions 4'' and 8'' (δ_H7.52d/ δ_C 124.8). The COSY correlations of these aromatic protons with the signal δ_H7.34t (2H), which also had a COSY correlation with δ_H7.34t (2H), 7.23t (1H) allowed the correct assign of the aromatic signals at 5''/7''/6'' positon (Figure 83).

The aliphatic chain was attributed due to the characteristic carbons at δ49.04 of a N-CH_2 (1''') group and at δ83.66 (d, *J*=164.80) of a carbon linked directly to a fluor atom. COSY correlations between each proton and its neighbour allowed for the correct attribution of signals 1''' to 5''' of the aliphatic chain (Figure 84).

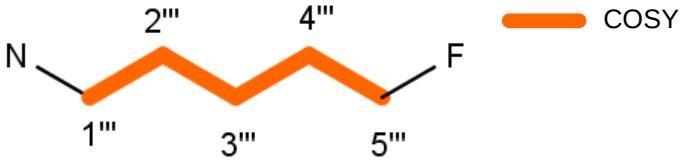


Figure 84 - Key COSY correlations in the aliphatic chain of SGT-25

Table 19 - ^{1}H and ^{13}C signals for SGT-25 in $CDCl_3$

Position	^{13}C, *J* (Hz)	^{1}H; *J* (Hz)
1	161.75	-
2'	137.80	-
3'a	122.76	-
3'	123.18	8.34, d, 1H, 8.0
4'	122.38	7.21, m, 1H
5'	126.68	7.39, m, 1H
6'	108.95	7.39, m, 1H
6'a	140.81	-
2''	55.75	-
2''a	29.56	1.87, s, 6H
3''	147.17	-
4''/8''	124.80	7.52, d, 2H, 8.0
5''/7''	128.38	7.34, t, 2H, 7.6
6''	126.53	7.23, m, 1H
1'''	49.04	4.40, t, 2H; 7.2
2'''	29.31	2.01, m, 2H,
3'''	22.65d, 5.0	1.48, m, 2H
4'''	29.86d, 19.7	1.75, m, 2H;
5'''	83.66d, 164.8	4.43, dt, 2H, 47.2; 6.0

The δ_C140.81 was assigned to C6'a by its HMBC correlation with H1'''. The HMBC correlation with the aromatic proton at δ_H8.34d with 3 quaternary carbons (δ_C140.81, 122.76 and 137.80) allowed the attribution of H3' (δ_H 8.34), C3'a (δ_C122.76) and C2' (δ_C137.8). The other 3 aromatic signals were assigned by COSY correlations. between H3' and H4' and also by HMBC correlations of H3' with C4'.

Analytical data from EI-MS and NMR allowed the confirmation of the unknown compound as SGT-25 and also allowed the addition of this compound to the EI-MS library of NPS. The ^{1}H NMR values are in accordance with the only published reference of this compound, although not assigned [94]. SGT-25 is a novel NPS and, there is a lack of analytical information available in the literature. Therefore, SGT-15 was analysed by NMR in different solvents: $CDCl_3$, benzene-d_6, DMSO and MeOD. ^{1}H and ^{13}C signals in all four solvents are shown in Table 20.

Table 20 - ^{1}H and ^{13}C NMR Signals of SGT-25 in $CDCl_3$, MeOD, Benzene-d_6 and DMSO

Position	$CDCl_3$	MeOD	Benzene-d_6	DMSO
	^{13}C, *J* (Hz)	^{13}C, *J* (Hz)	^{13}C, *J* (Hz)	^{13}C, *J* (Hz)
1	161.75	163.94	161.66	161.10
2'	137.80	138.59	139.00	137.28
3'a	122.76	123.79	123.65	121.99
3'	123.18	123.13	124.08	121.78
4'	122.38	123.66	122.73	122.28
5'	126.68	127.94	126.79	126.55
6'	108.95	110.93	109.07	110.35
6'a	140.81	142.41	141.24	140.58
2''	55.75	57.00	55.95	55.15
2''a	29.56	30.04	29.41	29.53
3''	147.17	148.51	147.91	147.71
4''/8''	124.80	125.91	125.32	124.78
5''/7''	128.38	129.36	128.60	128.03
6''	126.53	127.46	126.70	125.98
1'''	49.04	50.08	48.85	48.51
2'''	29.31	30.44	29.37	29.08
3'''	22.65, d, 5.0	23.70, d, 5.1	22.66, d, 5.0	22.07, d, 5.2
4'''	29.86, d, 19.7	31.00, d, 19.8	29.95, d, 19.8	29.34, d, 19.1
5'''	83.66, d, 164.8	84.67, d, 163.9	83.19, d, 166.1	83.67, d, 161.7

Position	$CDCl_3$	MeOD	Benzene-d_6	DMSO
	^{1}H, m *J* (Hz)	^{1}H, m, *J* (Hz)	^{1}H, m, *J* (Hz)	^{1}H, m, *J* (Hz)
1	-	-	-	-
2'	-	-	-	-
3'a	-	-	-	-
3'	8.34, d, 1H, 8.0	8.10, d, 1H, 8.2	8.84, d, 1H; 8.0	8.05, 1H, d, 8.4
4'	7.21, m, 1H	7.22, t, 1H, 8.0	7.03, t, 1H; 7.2	7.22, 1H, t, 7.6
5'	7.39, m, 1H	7.42, t, 1H, 7.5	7.09, t, 1H; 7.2	7.43, 1H, m
6'	7.39, m, 1H	7.61, d, 1H, 8.6	6.90, d, 1H; 8.4	7.78, 1H, d, 8.4
6'a	-	-	-	-
2''	-	-	-	-
2''a	1.87, s, 6H	1.81, s, 6H	1.76, s, 6H	1.73, 6H, s
3''	-	-	-	-
4''/8''	7.52, d, 2H, 8.0	7.48, d, 2H, 7.7	7.48, d, 2H, 7.6	7.43, 1H, m
5''/7''	7.34, t, 2H, 7.6	7.31, t, 2H, 7.5	7.16, m, 2H,	7.30, 2H, t, 7.6
6''	7.23, m, 1H	7.20, t, 1H, 8.0	7.06, m, 1H	7.19, 1H, t, 7.6
1'''	4.40, t, 2H; 7.2	4.51, t, 2H, 7.0	3.80, 2H, t, 7.1	4.52, 2H, t, 6.8
2'''	2.01, m, 2H,	2.01, m, 2H	1.50, 2H, m	1.93, 2H, m
3'''	1.48, m, 2H	1.44, m, 2H	1.00, 2H, m	1.37, 2H, m
4'''	1.75, m, 2H;	1.72, m, 2H	1.17, 2H, m	1.65, 2H, m
5'''	4.43, dt, 2H, 47.2; 6.0	4.40, dt, 2H, 47.5; 6.0	3.92, 2H, dt, 47.6; 6.0	4.42, 2H, dt, 47.5; 6.0

The analysis of 54 samples of herbal incenses (33 herbal incenses for the preliminary GC-EI-MS, 20 products for the second GC-EI-MS analysis and 1 seized sample) has allowed the construction of an in-house EI-MS library of 8 synthetic cannabinoids (JWH-018, JWH-122, JWH-210, AKB48, UR-144, XLR-11, MAM2201 and SGT-25). Again, the construction of an in-house library is of great importance for a routine laboratory, as the thorough study of the spectrometric and fragmentation behaviour of these compounds may aid in the discovery of novel NPS and in the continuous monitoring of already existing substances. This study has allowed for the identification of specific EI-MS fragments of some characteristic groups usually present in synthetic cannabinoids, with the advantage of having a R_t associated. The developed methodology allowed the characterisation of a recently reported compound in the EU, SGT-25, not yet reported in the literature.

From this analyses, only 12 synthetic cannabinoids were detected and analysed. However, more than 100 have already been reported in the EU [65].

3.3. Final In-House EI-MS Library of NPS

From products of the voluntary deliveries and seized samples, it was possible to obtain 12 synthetic cathinones (Figure 85 and Table 21) and 8 synthetic cannabinoids (Figure 86 and Table 22) used to build an in-house EI-MS library of NPS. This is very relevant, as, otherwise, these products would have probably ended up destroyed, meaning that this methodology allows the avail of street products, turning them into secondary standards.

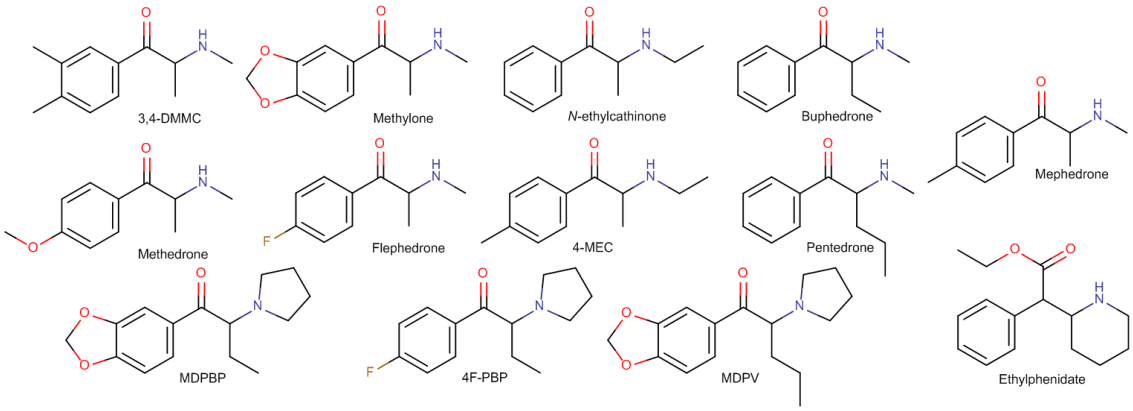


Figure 85 - EI-MS Library of Synthetic Cathinones

Table 21 - EI-MS Library of Synthetic Cathinones

Compound	R_t (min)	Base Peak	Other Relevant Fragmentations
3,4-DMMC	10.55	58	191 [M^+], 133, 105, 77
4F-PBP	11,25	112	233, 123, 95
4-MEC	9.81	72	191 [M^+], 119, 105, 91, 77, 65, 44, 39
Buphedrone	8.59	72	148, 105, 77, 57, 44
Ethylphenidate	12.56	84	146, 91, 77
Flephedrone	7.60	58	166, 123, 95, 75
Mephedrone	9.09	58	119, 105, 91
Methedrone	11.07	58	178, 135, 107
Methylone	12.14	58	207 [M^+], 149, 121, 42
MDPBP	15.21	112	260 [M^+], 232, 149, 121, 86, 55, 41
MDPV	15.67	126	273, 232, 149, 86, 65, 41
N-ethylcathinone	8.48	72	105, 77, 44
Pentedrone	9.55	86	105, 77, 44, 51, 39

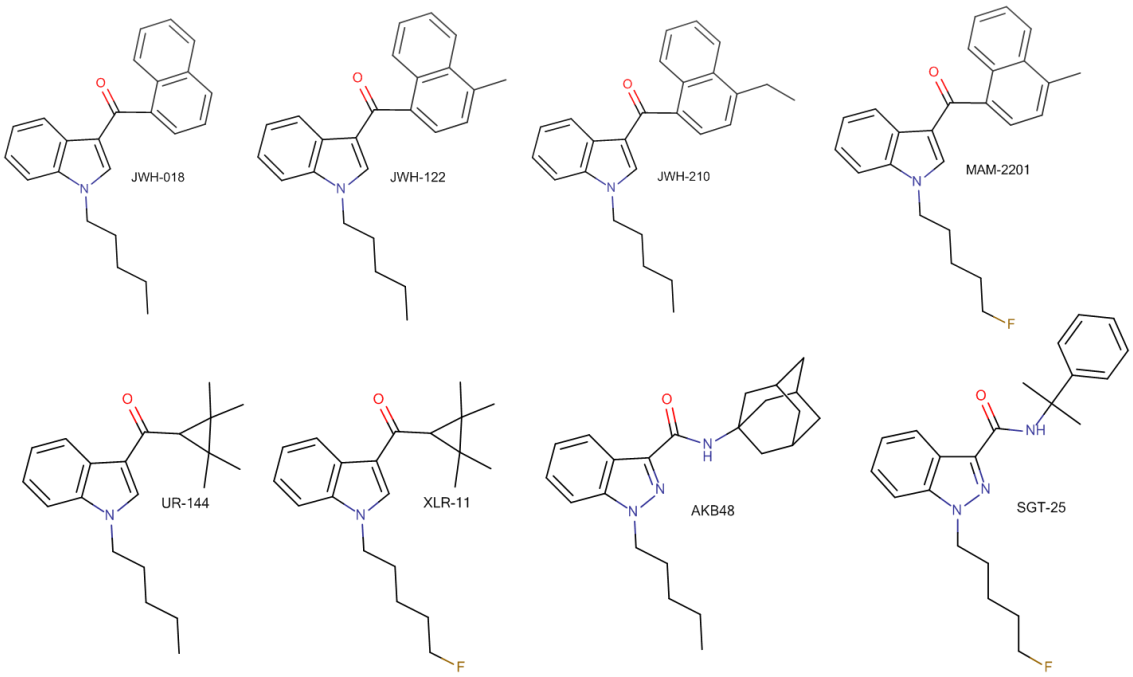


Figure 86 - EI-MS Library of Synthetic Cannabinoids

Table 22 - EI-MS Library of Synthetic Cannabinoids

Compound	R_t (min)	Base Peak	Other EI Fragmentations
JWH-210	16.2	369 [M^+]	312, 298, 214, 183, 144
JWH-018	14.1	341 [M^+]	284, 270, 214, 155, 144, 127
JWH-122	15.6	355 [M^+]	298, 284, 214, 169, 144, 141
MAM2201	17.2	373 [M^+]	298, 284, 232, 144, 141, 115
AKB-48	13.6	215	365 [M^+], 308, 294, 215, 145
XLR-11	8.3	232	329 [M^+], 314, 144
UR-144	7.0	214	311 [M^+], 144
SGT-25	11.1	233	367 [M^+], 352, 145

4. Conclusions

In the past decade, there has been an uprising on the availability and spread of NPS, substances commercialised with the intention to mimic the effects of illicit drugs, but that fall outside the scope of international and national drug control all over the world.

Since 2008, more than 450 NPS have been reported in the EU alone, making it a serious challenge for forensic practitioners to analyse these compounds, as appropriate methods and standards are either too expensive or hard to get. In Portugal, since 2013 that this topic has become of the greatest importance for forensic toxicologists, with the implementation of *Dec Lei* 54/2013, of 17th April, which forbids the commercialisation and production of about 149 substances, being liable to fast updates. Problem is that, with the implementation of the new legislation, analytical challenges were faced, regarding which methodology to use and the availability and cost of analytical standards. With the decree, more than 34000 products that were sold in smartshops in Portugal were delivered to the Portuguese police and a protocol was created between FCUL and LPC/PJ, with the objective to identify the compounds present in products in order to obtain standards for routine analyses.

Within the scope of this project, two types of products were analysed, plant feeders and herbal incenses, known to contain two of the most reported categories of NPS, synthetic cathinones and synthetic cannabinoids.

The methodology applied consists on the identification of these two types of products by NMR and EI-MS, allowing them to become secondary standards for analyses by GC-MS, as it permits a correct identification of the compounds present based not only on mass fragmentation, but also on different R_t for each compound.

The analysis of 115 samples of plant feeders (8 initial standards, 103 products from voluntary deliveries and 4 seized samples) has allowed the construction of an in-house EI-MS library of 12 synthetic cathinones (3,4-DMMC, 4-MEC, 4F-PBP, Buphedrone, Flephedrone, MDPBP, MDPV, Mephedrone, Methedrone, Methylone, *N*-ethylcathinone and Pentedrone) plus ethylphenidate. This is a case where the value of the developed methodology is shown, as it is rather difficult to distinguish between ethylphenidate and methylphenidate merely using GC-EI-MS (with no standards available); nevertheless, NMR allowed the correct characterisation of the compound as

ethylphenidate. The methodology used and the in-house library allowed the identification of a new synthetic cathinone in Europe, 4F-PBP, firstly suggested by MS comparison with the secondary standards and then confirmed by NMR analyses. The variability studies permitted the study of the qualitative and quantitative variations of synthetic cathinone in Portugal, showing no qualitative variance within lot number. Also, in the collection “Space Invader”, samples could be organised merely on lot numbers, without considering the brand name. An interesting remark is that, of the more of 70 synthetic cathinones already reported in the EU, only the 12 identified in the in-house library could be found in Portuguese powdered plant feeders from 2011 to 2013, plus ethylphenidate and caffeine.

The analysis of 56 samples of herbal incenses has allowed the construction of an in-house EI-MS library of 8 synthetic cannabinoids (JWH-018, JWH-122, JWH-210, AKB48, UR-144, XLR-11, MAM2201 and SGT-25). The developed methodology also allowed the characterisation of a recently reported compound in the EU, SGT-25, not yet reported in scientific journals. From the 134 synthetic cannabinoids already reported in the EU, only 12 (8 in the EI-MS library plus AM694, Cl-AM694 and AM1248) were detected in the 53 herbal incenses samples from Portuguese smartshops from 2013.

Concluding, from products of the voluntary deliveries and seized samples an EI-MS in-house library of NPS consisting of 21 synthetic compounds was created: 12 cathinones, 8 cannabinoids and ethylphenidate. The construction of this in-house library is of great importance for a routine laboratory, as the thorough study of the spectrometric and fragmentation behaviour of these compounds may aid in the discovery of novel NPS and in the continuous monitoring of already existing substances. The advantage of an R_t facilitates the correct identification on these compounds.This is very relevant, as, otherwise, these products would have probably ended up destroyed, meaning that this methodology allows the avail of street products,

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6. Appendix

Appendix I - Gaspar, H., Bronze, S, Ciríaco, S., **Queirós, C.R**., Matias, A., Rodrigues J., Oliveira, C., Cordeiro, C. and Santos, S., "*4F-PBP: 4F-PBP (4'-fluoro-α-pyrrolidinobutyrophenone), a new substance of abuse: structural characterization and purity NMR profiling"*, Forensic Science International, 252, pp. 168-176, 2015

Highlights

A new NPS, 4F-PBP was detected and identified for the first time in the EU.

Analytical data for 4F-PBP are reported for the first time.

Myo-inositol was found as cutting agent of 4F-PBP.

A qNMR method was applied to profile cathinones purity.

Abstract

The rapidly growing problem of new psychoactive substances (NPS) makes the time management for international control a real challenge, with the traditional detection methods becoming increasingly inadequate. NPS screening technologies, such as NMR, which allows multiple substances to be detected, characterized and quantified simultaneously from a single sample, offers a rapid solution to this problem. This study describes the application of NMR to the simultaneous detection, characterization and quantification of samples of white powders seized by the Portuguese Police. 4F-PBP (4′-fluoro-α-pyrolidinobutyrophenone) a new synthetic psychoactive cathinone cut with *myo*-inositol was found in two seized products. The structural characterization of 4F-PBP was elucidated in the mixture, and confirmed after isolation from the matrix by ^{1}H, ^{13}C, ^{19}F NMR and MS. *Myo*-inositol was found for the first time as a cutting agent of cathinones. Furthermore another seized product was characterized as being MDPBP, with a high degree of purity, and its spectroscopic elucidation enabled the correction of ^{13}C NMR literature assignments.

Keywords

Designer drugs; Cathinones; Pyrolidinophenone; 4F-PBP; Fluoropyrolidinobutyrophenone; Quantification; NMR

Appendix II - Leal, C., Lopes, R., Matias, A., Rodrigues, J. and Gaspar, H., "Identification of Synthetic Cathinones in Plant Feeders", *II Jornadas Ibéricas de Toxicologia, 13-15 November 2014, Faculdade de Ciências da Saúde, Universidade da Beira Interior*, Covilhã, Portugal (Poster)

In the past few years, there has been an uprising of new psychoactive substances (NPS) available in Smartshops and over the Internet. These NPS may be masked in herbal mixtures or may be presented as tablets, capsules or in powdered forms, being suitable to inhale, smoke, swallow or chew. The sold products often come with the caveat of not being for human consumption and are marketed as herbal incenses, bath salts or plant feeders. Recently, a new decree has been implemented in Portugal (Dec-Lei 54/2013 de 17 de Abril), which forbids the production and commercialisation of about 159 NPS, being liable to fast updates, in order to keep up with the everyday appearance of new substances.

The fast appearance of new substances and the lack of adequate analytical standards turned the analysis of NPS by forensic scientists into a challenge. This project is being developed within the scope of the collaboration between the *Faculdade de Ciências da Universidade de Lisboa* (FCUL) and the *Laboratório de Polícia Científica da Polícia Judiciária* (LPC/PJ), as a result of the need to create effective analytical databases that will facilitate the quick identification of these drugs in a forensic context. Therefore, the goal of this project is to develop an analytical methodology that allows the identification of these substances in different products, even in the absence of standards.

In this context, we started compiling a library of NPS mass spectra from the products being sold in the market of drugs of abuse in Portugal. NMR and GC-MS analysis of several plant feeder samples allowed the identification of different synthetic cathinones, as seen in literature (Araújo et al, 2014 and Zancajo et al, 2014).

In 29 samples of Blast, Bliss and Blow commercial products in circulation in Portugal before the decree, analysed by GC-MS, 5 different compounds have been identified. In the future, more compounds will be identified, characterised and added to the mass spectra library, so as to allow for the rapid identification of new products that may emerge in the still active market of "legal highs".

Keywords: NPS; Plant Feeders; NMR; GC-MS

Appendix III - Leal, C., Ciríaco, S., Matias, A. and Gaspar, H. “Characterisation of Plant Feeders”, 3rd International Meeting on Forensic Science and Criminal Behaviour - Globalization of Crime, 8-9 May 2015, Instituto Superior de Ciências da Saúde Egas Moniz, Almada, Portugal (Oral)

In the past years, there has been an uprising of new psychoactive substances (NPS) available in Smartshops and over the Internet. These NPS may be presented as tablets, capsules or in powdered forms, being suitable to inhale, smoke, swallow or chew. The sold products often come with the caveat of not being for human consumption and are marketed as bath salts or plant feeders.

Recently, a new decree has been implemented in Portugal (Dec-Lei 54/2013 de 17 de Abril), which forbids the production and commercialisation of about 159 NPS, being liable to fast updates, in order to keep up with the everyday appearance of new substances.

The fast appearance of new substances and the lack of adequate analytical standards turned the analysis of NPS by forensic scientists into a challenge. This project is being developed within the scope of the collaboration between FCUL and LPC/PJ, as a result of the need to create effective analytical databases that will facilitate the quick identification of these drugs in a forensic context. Therefore, the goal of this project is to develop an analytical methodology that allows the identification of these substances in different products, even in the absence of standards.

I n this context, we started compiling a library of NPS mass spectra from the products being sold in the market of drugs of abuse in Portugal. NMR and GC-MS analysis of several plant feeder samples allowed the identification of different synthetic cathinones, as seen in literature (Araújo et al, 2014 and Zancajo et al, 2014).

This methodology has allowed for the differentiation of compounds with similar chromatographic and ionization behaviours, but, more importantly, has allowed the identification and characterisation of a new substance, 4F-PBP (4’-fluoro-α-pyrolidinobutyrophenone), from samples of white powders seized in Portugal

In the future, more compounds will be identified, characterised and added to the mass spectra library, so as to allow for the rapid identification of new products that may emerge in the still active market of “legal highs”.

Appendix IV - Queirós, C., Gonçalves, C., Ciríaco, S., Matias, A., Rodrigues, J. and Gaspar, H., "NMR Characterisation of SGT-25: A New Psychoactive Substance", *19th European Symposium on Organic Chemistry, Faculdade de Ciências da Universidade de Lisboa,* 12-16 July 2015, Lisboa, Portugal (Poster)

In the past few years, there has been an uprising of new psychoactive substances (NPS) available in Smartshops and over the Internet. These NPS have been separated in different categories, either according to their structure, like synthetic cathinones, or according to their biological activity, like synthetic cannabinoids. Over the past years, more than 450 new NPS have been reported to the Early Warning System by EU Member States. In 2014 alone, more than 100 substances were reported. Up to that date, more than 130 of the reported substances were synthetic cannabinoids. These compounds are characterised by its affinity to the CB1 and CB2 receptor sites, similar to Δ9-THC, the compound present in cannabis.

Two years ago, a Decree-Law was published in Portugal, which forbids the production and commercialisation of about 159 NPS, 46 of which are synthetic cannabinoids, being liable to fast updates, in order to keep up with the everyday appearance of new substances.

I n order to circumvent the new legislation, compounds that fall outside the list of prohibited substances may arise and their rapid identification is of the uttermost importance in forensic laboratories. However, the rapidly growing problem of NPS makes the time management for international control a real challenge, with the traditional detection methods becoming increasingly inadequate. Nuclear Magnetic Resonance (NMR) spectroscopy offers a rapid solution to this problem as it allows the identification of a compound, even in a mixture, without the need of the analyte standard. Using this methodology we have recently characterised a new synthetic cathinone, 4F-PBP and reported it for the first to the European Union Early Warning System.

This study describes the application of NMR in the characterisation of SGT-25 (1-(5-fluoropentyl)-N-(1-methyl-1-phenylethyl)-1H-indazole-3-carboxamide), a novel 'third generation' synthetic cannabinoid, from a sample seized in Portugal, which is not part of the list of controlled NPS from the new Decree-Law

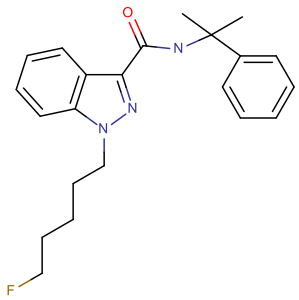

Figure 87 - Chemical Structure of SGT-25

This compound was firstly synthesised with the aim to be used as medication in animals and humans, due to high affinities for the cannabinoid CB1 and CB2 receptor sites.

The structural characterisation of SGT-25 was elucidated in the seized sample and confirmed after isolation using a liquid chromatography system by 1D and 2D NMR techniques, in different solvents ($CDCl_3$, benzene (D_6), MeOD and DMSO) and also by GC-MS. This is a newly detected compound in Portugal and has only recently been detected in the EU (first report from November 2014).

Appendix V - Leal, C., Gonçalves, C., Matias, A., Rodrigues, J. and Gaspar, H. “Spice in Portugal: a source of NPS standards”, *7th European Academy of Forensic Sciences Conference,* 6-11 September 2015*,* Prague, Czech Republic (Poster)

In the past few years, there has been an uprising of new psychoactive substances (NPS) available in Smartshops and over the Internet. These NPS may be masked in herbal mixtures or may be presented as tablets or in powdered forms, being suitable to inhale, smoke or swallow. The sold products often come with the caveat of not being for human consumption.

Recently, a new decree has been implemented in Portugal (Dec-Lei 54/2013 de 17 de Abril), which forbids the production and commercialisation of about 159 NPS, being liable to fast updates, in order to keep up with the everyday appearance of new substances. The fast emergence of new substances and the lack of adequate analytical standards turned the analysis of NPS by forensic scientists into a challenge.

This project is being developed within the scope of the collaboration between the *Faculdade de Ciências da Universidade de Lisboa* (FCUL) and the *Laboratório de Polícia Científica da Polícia Judiciária* (LPC/PJ), as a result of the need to create effective analytical databases that will facilitate the quick identification of these drugs in a forensic context in Portugal.

In this context, 30 herbal incenses available in Portuguese Smartshops in the period prior to the legislation were analysed by GC-MS and scanned against existing MS spectra databases. From this analysis, 10 synthetic cannabinoids (JWH-210, JWH-018, JWH-122, JWH-250, MAM2201, AKB48, UR-144, UR-144-cyclopropyl rearrangement product, AM-694, AM-1248), one synthetic cathinone (MDPV) and one tryptamine (5-MeO-DALT) were identified.

In order to obtain validated standards for GC-MS analysis, some synthetic cannabinoids from the JWH family were isolated through liquid chromatography and identified by NMR. This led to the creation of an in-house MS spectra database based on retention times and mass fragmentation of the purified compounds. In the future, more compounds will be identified, characterised and added to the mass spectra library, so as to allow for the rapid identification of new products that may emerge in the still active market of “legal highs”.

Appendix VI - Gaspar, H., Ciríaco, S., **Leal, C**., Matias, A., Rodrigues, J. e Santos, S., "Tracking NPS: NMR for a rapid identification of new substances", Lisbon Addictions 2015, 23-25 September 2015, Lisbon, Portugal (Poster)

In the past years, there has been an uprising of new psychoactive substances (NPS) available in "smartshops" and over the Internet. NPS are defined as "new narcotic or psychotropic drugs that are not listed in the Single Convention on Narcotic Drugs of 1961 or the Convention on Psychotropic Substances of 1971, but which may pose a public health threat comparable to that posed by substances listed in those conventions". These NPS may be masked in herbal mixtures or presented as tablets, capsules or powdered forms, being suitable to inhale, smoke, swallow or chew. The sold products often come with the caveat of not being for human consumption and are marketed as herbal incenses, bath salts or plant feeders.

Recently, a new decree has been implemented in Portugal (Dec-Lei 54/2013 de 17 de Abril), which forbids the production and commercialisation of about 159 NPS, being liable to fast updates, in order to keep up with the everyday appearance of new substances.The rapidly growing problem of NPS makes the time management for international control a real challenge, with the traditional detection methods becoming increasingly inadequate. Nuclear Magnetic Resonance (NMR) spectroscopy offers a rapid solution to this problem as it allows the simultaneous characterization and quantification of compounds from a single sample without the need of chemical standards.

The present work was developed within the scope of the collaboration between the *Faculdade de Ciências da Universidade de Lisboa* (FCUL) and the *Laboratório de Polícia Científica da Polícia Judiciária* (LPC/PJ), and describes the application of NMR in the simultaneous detection, characterization and quantification of 4F-PBP (4'-fluoro-alpha-pyrolidinobutyrophenone), a new designer drug, together with the cutting agent *myo*-inositol, from samples seized in Portugal. The structural characterization of 4F-PBP was elucidated in the mixture, and confirmed after isolation from the matrix by different analytical techniques (^{1}H, ^{13}C, ^{19}F NMR, GC-MS and FTICR). 4F-PBP is a newly detected substance in the European Union, reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) for the first time in late January (Gaspar et al 2015).The methodology used has proven to be effective for the

characterization and quantification of NPS, being a rapid tool for the identification of new emerging substances.

To the authors knowledge, this is the first time that inositol is reported as a cutting agent in a seizure of synthetic cathinones and, more importantly, in a very high percentage (around 60%). This fact can be a hint that in Portugal, like in other countries, after illegalization of this kind of substances, there is a downtrend in the purity of the products. This decrease in purity may lead to serious health consequences, with unpredictable dangerous effects, since consumers used to products with low purity can easily overdose when using a pure sample.